ISOFLAVONE EFFECTS ON INSULIN LIKE GROWTH FACTORS AND BREAST CANCER

by

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SIGNED DECLARATION

I, Maeli Campbell-McNulty, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:

Date: 14TH MARCH 2008

ABSTRACT

An increased concentration of insulin-like growth factor-1 (IGF-1) is recognized to be an independent risk factor for pre-menopausal breast cancer. Tamoxifen is thought to initially reduce IGF-1 concentrations and increase levels of the IGF binding proteins 1 and 3. Isoflavones are oestrogen-like plant compounds, which may, because of their structural similarities to tamoxifen, also alter IGF status.

This thesis first compares IGF-1, IGF binding protein 1(BP-1) and IGF binding protein 3 (BP-3) levels in breast cancer patients (n=14) compared with control subjects (n=23) and then assesses the effect of tamoxifen on IGF status after 9, 18 and 27 months of treatment. Concentrations of IGF-1, BP-1 and BP-3 at baseline did not differ between cases and controls. However on tamoxifen treatment, BP-1 and BP-3 were both significantly increased after 18 and 27 months while the IGF-1/BP-3 ratio was significantly decreased after 9 and 18 months.

A feasibility study was then conducted to compare the effects of acute (single 80 mg load) versus chronic (80 mg/day for 7 days) administration of isoflavone-containing soy and linseed cereal bars on IGF-1, IGFBP-1 and IGFBP-3 in healthy female volunteers (n=10). Assays were established for the analyses of serum IGF-1, BP1 and BP3 concentrations and GCMS analysis of isoflavones in urine. Concentrations of IGF-1 were unchanged following the single 80 mg load. However, IGF-1 and BP-3 concentrations were significantly elevated after a week of supplementation with the soy and linseed bar.

A larger study was then carried out to assess the effect of one-month isoflavone supplementation (80 mg/d) in tablet form, on IGF status in healthy pre- (n=16) and post-menopausal (n=7) women. This was a randomized, placebo-controlled

crossover study with a minimum two-month washout period. Hormonal, antioxidant and lipid altering effects of the supplement were also examined, as was background diet. For pre-menopausal subjects, while there was an effect of time on IGF-1 ($p=0.005$), BP-1 ($p=0.004$), and BP-3 ($p<0.001$) confirming that IGF profile is influenced by menstrual cycle, this did not differ between placebo and isoflavone supplement. When the change in IGF-1 over the whole supplementation period was compared between the supplement and placebo phases, there was a non-significant reduction in change in IGF-1 ($p=0.06$) on isoflavone supplement compared to placebo. However, this may have been due to the non-significantly higher baseline concentrations of IGF-1 during the supplement phase. In post-menopausal subjects, there was no effect of isoflavone supplementation in comparison with placebo on IGF-1, BP-1 or BP-3. Finally, experiments were done *in vitro* to study the effect of isoflavones on DNA synthesis and proliferation in ERα positive MCF-7 and ERα negative MDA-MB 231 human breast cancer cells. In MCF-7 cells, low concentrations of isoflavones (0.1μM-10μM) stimulated DNA synthesis while high concentrations of genistein and equol were able to inhibit DNA synthesis with IC50 values of 93μM and 55μM respectively compared with 1.17μM tamoxifen. In MDA-MB231 cells the Isoflavones did not stimulate DNA synthesis at any concentrations but significantly inhibited DNA synthesis with IC50 values of 114μM, 14.1μM and 14.55μM for daidzein, genistein and equol respectively compared with 1.35μM tamoxifen.

This work suggests that isoflavone supplementation may alter IGF profile in pre-menopausal women, and could suggest a role for these dietary compounds in breast cancer prevention. This should be further investigated in long term intervention studies with isolated isoflavone supplements.

For my parents

PUBLICATIONS ARISING FROM WORK CONTRIBUTING TO THIS THESIS

Campbell, M.J., Woodside, J.V., Secker-Walker, J., Titcomb, A. & Leathem, A.J. (2001). IGF status is altered by tamoxifen in patients with breast cancer. *Mol Pathol*, **54,** 307-10.

Campbell, M.J., Woodside, J.V., Honour, J.W., Morton, M.S. & Leathem, A.J. (2004). Effect of red clover-derived isoflavone supplementation on insulin-like growth factor, lipid and antioxidant status in healthy female volunteers: a pilot study. *Eur J Clin Nutr*, **58,** 173-9.

Woodside, J.V. & Campbell, M.J. (2001). Isoflavones and breast cancer. *J Br Menopause Soc*, **7,** S17-S21.

Woodside, J.V., Campbell, M.J., Denholm, E.E., Newton, L., Honour, J.W., Morton, M.S., Young, I.S. & Leathem, A.J. (2006). Short-term phytoestrogen supplementation alters insulin-like growth factor profile but not lipid or antioxidant status. *J Nutr Biochem*, **17,** 211-5.

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CHAPTER ONE – INTRODUCTION

Breast cancer is one of the most important public health problems facing women in the UK and other Western populations today. With over 40,000 new cases diagnosed in the UK in 2000 alone, the disease accounts for 17% of female cancer mortality with almost 13,000 deaths in UK each year (Toms, 2004). Incidence rates have increased by 70% since 1971 with one in ten women developing breast cancer at some point in their lives (ONS, 31 October 2003). Breast cancer is a complex, multifactorial disease, but established risk factors include nulliparity, late first pregnancy, early menarche, late menopause, oral contraceptive use, hormone replacement therapy (HRT), obesity, increased alcohol consumption, exposure to ionising radiation and inheritance of mutations in the BRCA1 and BRCA2 genes (McPherson et al., 2000).

1.1 Role of dietary factors

There is strong epidemiological evidence that environmental factors such as diet have a role in breast cancer aetiology (Miller et al., 1994). This evidence initially came from population and migrant studies where Chinese, Japanese or Filipino's who had lived in the United States for a decade or longer had a risk 80% higher than more recent migrants (Ziegler et al., 1993). The timing of immigration to the US was also important in determining breast cancer risk and when migration occurred later in life, rates for breast cancer were substantially lower than when migration occurred early, although they were still much higher than in the homeland populations (Hunter & Willett, 1996; Shimizu et al., 1991).

Subsequently, cohort and case-control studies have been carried out, mainly focussing on the hypothesis that a diet rich in fat can increases the risk of breast cancer (Hunter & Willett, 1996). Assessing this relationship is complicated and study design, dietary methodology and choice of study population is important (Miller et al., 1994). The results of prospective studies have largely been equivocal (Hunter & Willett, 1996), while fat-reducing intervention studies have shown some effects on total and free oestradiol in post-menopausal but not pre-menopausal women (Bingham et al., 1998). Other studies suggest that olive oil may be protective for its high levels of monounsaturated fatty acids and/or phenolic compounds (Hashim et al., 2005). However recent findings from large prospective studies in the UK and Europe (EPIC) suggest that risk of breast cancer is associated with intake of saturated fat (HR 1.22, 95% CI 1.06-1.4), with 35g /day vs 10g/day approximately doubling risk (Bingham et al., 2003) in peri and post-menopausal women studied. While total or specific fruit and vegetable intake was not found to be associated with risk of breast cancer in pre or postmenopausal women in the initial analysis of data in women across 10 countries in Europe (van Gils et al., 2005), the UK women's cohort study (n= 35,792) has found that for pre-menopausal women, total fibre is significantly protective against breast cancer, especially fibre from cereals and possibly fruit (Cade et al., 2007), while high consumption of meat, especially processed meat, significantly increased risk of breast cancer (Taylor et al., 2007). It is possible that specific components of dietary fibre such as lignans present in seeds and wholegrains are responsible for reducing risk of breast cancer (McCann et al., 2004). There is also interest in the protective effect of other dietary factors, such as vitamins, other micronutrients and phytochemicals (Bingham et al., 1998).

1.2 Isoflavones

Plants contain a large number of potential antioxidant/anticancer type compunds, known as phytoestrogens (Bingham et al., 1998), many of which can be classed as phenols, as they contain an active benzene ring with a hydroxyl group attached. An example of such compounds is the flavonoid family which are present in fruit and vegetables as the biologically inactive phenolic glycosides. The aglycone (sugar free) part of the compund is characterised by a C6-C3-C6 backbone. Sub groups such as Isoflavones and Flavanols have been classified to encompass differences in the position of ring B and varying degrees of oxidation and saturation of the pyrone ring **(Figure 1.2.1)**. Isoflavones are found predominantly in legumes (peas, beans, lentils, etc). Soybeans and soy products are perhaps the most common food source of isoflavones and contain large amounts of the isoflavones genistein and daidzein (Coward et al., 1993). But other legumes, especially clovers, contain higher total levels and, in addition to genistein and daidzein, also contain formononetin and biochanin (Francis et al., 1967). Interest has focused on isoflavones and breast cancer due to the relatively low breast cancer mortality rates in Asian countries where soy foods are commonly consumed. In Japan and China, for example, the breast cancer mortality rate is about one quarter of that in the United Kingdom (Society, 2006). In addition, a lower proportion of breast biopsies from Asian women contain hyperplasia, atypical hyperplasia, and apocrine metaplasia than breast biopsies from American women (Sasano et al., 1975; Schuerch et al., 1982). The evidence linking isoflavone or soy intake with breast cancer risk comes from a number of sources and these will be considered in turn.

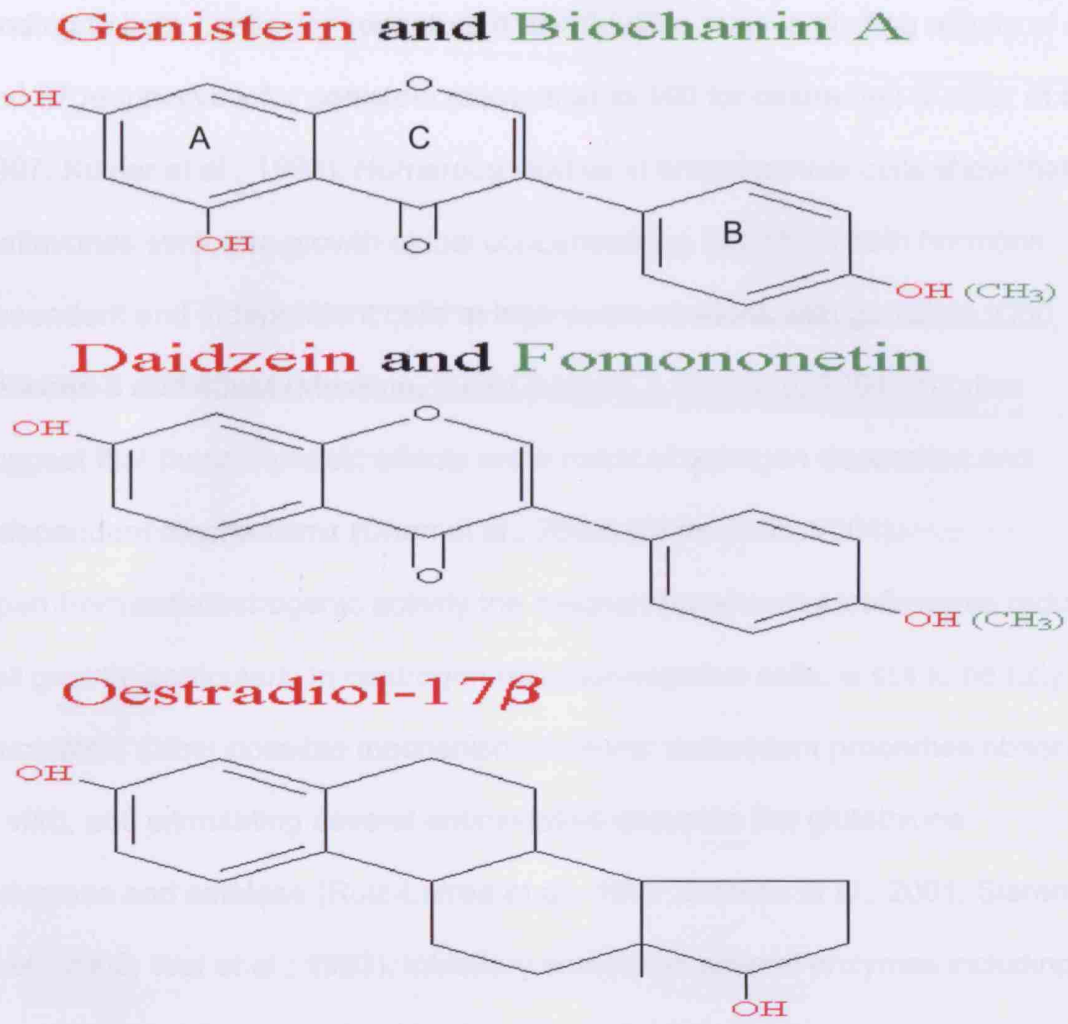


Figure 1.2.1: Chemical structure of isoflavones and oestradiol.

Basic structure comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (c) in the middle

1.2.1 *In vitro* evidence

Isoflavones have been considered weak oestrogenic compounds *in vitro*, binding to both oestrogen receptors α and β with a relative binding affinity of 4 and 87 respectively for genistein (compared to 100 for oestradiol) (Kuiper et al., 1997; Kuiper et al., 1998). Numerous studies in breast cancer cells show that Isoflavones stimulate growth at low concentrations but inhibit both hormone dependent and independent cells at high concentrations with genistein IC50 between 5 and 40μM (Messina, 1999) (Magee & Rowland, 2004). Studies suggest that these biphasic effects are a result of estrogen dependent and independent mechanisms (Chen et al., 2003) (Chen et al., 2004).

Apart from anti-oestrogenic activity the mechanism whereby isoflavones reduce cell growth, particularly in oestrogen receptor-negative cells, is still to be fully elucidated. Other possible mechanisms include: antioxidant properties observed *in vitro*, and sitmulating several antioxidative enzymes like glutathione reductase and catalase (Ruiz-Larrea et al., 1997; Sierens et al., 2001; Sierens et al., 2002; Wei et al., 1993), inhibitory actions on several enzymes including aromatase and those involved in signal transduction, like tyrosine protein kinases (Akiyama et al., 1987), MAP kinase (Thorburn & Thorburn, 1994) and ribosomal S6 kinase (Linassier et al., 1990). Genistein has been shown to inhibit the activity of DNA topoisomerase I and II (Constantinou et al., 1990), inhibit angiogenesis (Fotsis et al., 1995) and metastasis (Li et al., 1999), increase apoptosis (Shao et al., 1998a) (Sarkar & Li, 2003), increase the *in vitro* concentrations of transforming growth factor β (Kim et al., 1998) and increase protein and mRNA expression of the IGF-1 receptor and IRS-1 (Chen & Wong, 2004). Genistein can also regulate signalling pathways involved in cell proliferation (NFKB and AKT) (Davis et al., 1999; Li & Sarkar, 2002), and has

recently been shown to increase expression of all genes involved in cell cycle control apoptosis and DNA repair including BRAC1, p53, p21, Rad51, BARD, Bax GADD45 and RF1 (Caetano et al., 2006). Genistein can also regulate many genes for which roles are still being discovered (Barnes, 2004; Naciff et al., 2002). It is also possible that phytoestrogen effects are mediated via their action on growth factors and growth factor binding proteins (Adlercreutz, 2002a; Chen & Wong, 2004) with vegetarian diets rich in phytoestrogens associated with low plasma concentration of insulin, insulin Like Growth Factor-1 (IGF-1) and higher levels of IGF-1 binding proteins and SHBG (Giovannucci, 1999). Although most of these studies have been carried out with genistein, daidzein also exhibits anti-cancer properties (Scholar & Toews, 1994), although it has received less attention (Jing et al., 1993), and biochanin produced greater growth inhibition than genistein in MCF-7 breast cancer cells (Peterson et al., 1996) and has potent anti-mutagenic activity (Chae et al., 1991). The metabolite equol has also received greater attention recently with superior oestrogenic and antioxidant activity (Setchell et al., 2002).

In vitro studies suggest that physiological levels of oestrogen may also impact on the effect of isoflavones *in vivo* (Messina et al., 2006) and some findings suggest that the clinical relevance of these genomic and non-genomic effects found *in vitro* may not actually be significant (Santell et al., 2000). Thus caution is warranted in the interpretation of some of these studies because some of the effects may have been observed at higher concentrations than would be found *in vivo.*

1.2.2 Animal evidence

Approximately 70% of early studies in rat and mice models (Barnes, 1995) have found that feeds of soy protein or whole soy beans had protective effects, lowering the number of carcinogen-induced tumours when compared with standard diets. Isoflavone-depleted soy was found to be inactive in these models (Barnes, 1995). A recent review (Messina et al., 2006) also found that the majority of rodent studies showed inhibition of tumour development with soy or isoflavone supplementation prior to carcinogen- induced tumours (Magee & Rowland, 2004) (Constantinou et al., 2001; Yan et al., 2002) or implanted cancer cells (Gallo et al., 2006; Zhou et al., 2004). However there were exceptions to this (Allred et al., 2004a; Cohen et al., 2000; Day et al., 2001; Thomsen et al., 2005) and a review (Cooke, 2006) suggests that isoflavones have limited benefit once cancer is established in animal models.

Studies using overectomized mice injected with MCF-7 cells and implanted with estrogen pellets found that diets rich in genistein (Hsieh et al., 1998), isoflavone rich soy protein (Allred et al., 2001), or isoflavone extracts (Allred et al., 2004b) stimulated tumour growth compared with standard diets once implanted estrogen pellets were removed. Although this model is more inclined to show growth with weak estrogenic compounds, dietary genisten was still able to stimulate tumour growth in the presence of low levels of plasma estradiol mimicking conditions in postmenopausal women (Ju et al., 2006a).

The outcome of these studies are not only affected by the model used, but the type of soy product (Allred et al., 2004b; Constantinou et al., 2001) and specific isoflavone, with equol showing no stimulation of tumour growth when compared to daidzein (Ju et al., 2006b). Others have found that combinations of isoflavones with green/black tea (Zhou et al., 2004) or flaxseed (Thompson &

Ward, 2005) give even greater benefit than either alone. Furthermore, the timing of exposure to the carcinogen compared with ovarectomy and isoflavone intake can also affect outcomes, with genistein increasing tumours under certain conditions (Allred et al., 2004a) (Ueda et al., 2003).

Stuides exposing pre-pubertal rats to genistein (Cotroneo et al., 2002; Lamartiniere et al., 2002a; Lamartiniere et al., 1995; Lamartiniere et al., 2002b) also reduced the number of tumours later induced by administering DMBA carcinogen to adults. It is thought that in this model genistein promotes cellular differentiation thereby causing breast epithelial cells to be less vulnerable to carcinogen initiation. Other studies have also shown that subcutaneous injection of genistein in newborn rats can protect against DMBA induced tumours while increasing tumour resistant biochemical markers (Cabanes et al., 2004; Hilakivi-Clarke et al., 1999; Lamartiniere et al., 1995).

Certainly there are limitations with rodent studies and the large quantities of equol produced by rat gut flora vs human should be considered when using rodent models given that equol's oestrogenic activities are comparable to genistein but with preferential binding to Erβ (Cooke, 2006; Gu et al., 2006; Setchell et al., 2002; Setchell et al., 2005).

1.2.3 Epidemiological evidence

1.2.3.1 Soy/Isoflavone intake

Although there is supportive evidence from *in vitro* and animal studies that isoflavone intake may influence the risk of breast cancer, this clearly requires confirmation in human studies. Epidemiologic studies indicate that in populations with a high isoflavone intake the risk of breast cancer is reduced (Adlercreutz, 1990; Ingram et al., 1997; Wu et al., 1998), and that within ethnic

groups whose diets are traditionally high in isoflavone intake, change in diet due to adoption of western lifestyle has led to an increase in breast cancer incidence (Buell, 1973; Lee et al., 1988).

Epidemiological studies of human dietary soy intake and breast cancer risk have recently been reviewed (Adlercreutz, 2002a; Adlercreutz, 2002b; Peeters et al., 2003) and include case control and prospective studies **(Table 1.2.3.1)**. To date, case control studies have assessed intake by dietary questionnaire (Hirose et al., 1995; Lee et al., 1991; Witte et al., 1997; Wu et al., 1996; Yuan et al., 1995) (Dai et al., 2001; Hirohata et al., 1985; Shu et al., 2001) and/or plasma/urine concentrations (Adlercreutz et al., 1986; Adlercreutz et al., 1982; Ingram et al., 1997; Pietinen et al., 2001). Those that have assessed intake through dietary questionnaires include the following:

Of studies conducted in China, (Yuan et al., 1995) found no association between intake of soy and risk of breast cancer in either pre or post menopausal women but did see a strong protective effect from fibre rich foods, while (Dai et al., 2001) did find a dose-response relation between soy food intake and breast cancer risk in women with high BMI (p=0.02). (Shu et al., 2001) found that soy intake during adolescence (age 13-15) was associated with lower risk of breast cancer in later life.

In Singapore, high intake of soy was associated with a reduced risk in pre-menopausal women but not in post-menopausal women (Lee et al., 1991) (Lee et al., 1992). In pre-menopausal women, high daily consumers ($\geq$55.0 g soy product) showed a 60% (95% CI 0.2, 0.9) reduced risk compared with low daily consumers (<20.3 g). Similarly, in Japan, a high intake of soybean curd (tofu) was associated with a statistically non-significant reduced risk of breast cancer in pre-menopausal but not in post-menopausal women. However, in both

groups of women, there was no association between intake of miso soup, which was categorised in the food frequency questionnaire as daily or occasional-to-never, and breast cancer risk (Hirose et al., 1995). A recent smaller case control study in Japan, by the same author, found a statistically significant inverse association between tofu or isoflavone intake and risk of breast cancer in Japanese pre-menopausal women, but again this was not found with post-menopausal women (Hirose et al., 2005).

A study In the USA and Canada (Witte et al., 1997) found that tofu intake was associated with lower risk of pre-menopausal bilateral breast cancer. Among Asian Americans, intake of tofu was associated with a lower risk of breast cancer both overall and separately in pre- and post-menopausal women. Similar results were obtained after adjustment for relevant menstrual and reproductive factors and selected dietary factors (Wu et al., 1996). Asian American women in the highest intake group (>120 times/y) showed a 30% reduced risk (95% CI 0.4, 1.0) compared with women in the lowest intake group (<12 times/y). Analysis by migrant status showed a significant protective effect in migrants but not in Western-born Asian Americans. Another case control study in a similar population of Asian Americans found that the risk of breast cancer was significantly inversely associated with soy intake during adolescence and adult life [OR=0.53, 95% CI=0.36-0.78], with women who reported soy intake at least once per week during adolescence showing a statistically significantly reduced risk of breast cancer (Wu et al., 2002). A recent case control study in Canada restrospectively assessed phytoestrogen intake during adolescence of breast cancer patients vs controls and also found that higher phytoestrogen intake (both isoflavones and lignans) during adolescence was associated with reduced risk of breast cancer, with a trend observed from the lowest to the highest

quartile [OR =0.91, 95% CI= 0.79-1.04, OR=0.71, 95% CI=0.62-0.82, p trend<0.001] (Thanos et al., 2006).

Recently a large case control study in Italy analysed intake of the six principal classess of flavonoids and found that only increased intake of flavones gave a significant reduced risk of breast cancer [OR=0.81, highest vs lowest quintiles, p-trend=0.02] (Bosetti et al., 2005). This appears to be in line with an earlier case control study done in Greece (Peterson et al., 2003).

Case Control studies that have assessed intake by plasma or urine concentrations include the following:

Two studies in Australia have looked at levels of isoflavones or their metabolites in biological fluids. (Ingram et al., 1997) carried out a case-control study of 144 breast cancer patients and an equal number of age-matched healthy controls in Australia. When examining isoflavone and metabolite excretion in quartiles, as equol excretion increased, the relative risk of breast cancer was significantly reduced. The risk for the highest quartile of excretion of equol, after adjustment for confounding variables, was one quarter that of the lowest quartile. Similar effects were seen with respect to the lignan, enterolactone. Daidzein showed a similar pattern, but the trend was not significant. Unfortunately, the laboratory assay for genistein was not reliable enough to report results. These results were surprising as the total phytoestrogen intake of the study subjects, as estimated from the urinary phytoestrogen excretion, was very low (Messina et al., 1997).

A similar, preliminary study, also carried out in Australia but this time in post-menopausal women and controls, found that those with breast cancer had lower 24-h urinary daidzein excretion than controls (p=0.03) and a trend towards lower genistein (p=0.08) (Murkies et al., 2000). A study in China (Zheng et al., 1999) showed that breast cancer cases had substantially lower levels of

isoflavones in urine than age matched controls, while studies in Finland (Adlercreutz et al., 1986; Adlercreutz et al., 1982; Pietinen et al., 2001) have also found lower urinary and plasma enterolactone concentrations in women with breast cancer vs controls.

Two recent nested case control studies examining phytoestrogen concentrations from women participating in the large European Prospective Investigations into cancer (EPIC) have had conflicting results. In the UK population, where 333 women who developed breast cancer, higher equol and daidzein in serum and urine significantly increased breast cancer risk (Grace et al., 2004). In this population the majority of women were post-menopausal, although analysis was combined. Analysis of the Dutch cohort of 383 women who developed breast cancer found that high levels of all isoflavones were associated with lower breast cancer risk and this was significant for plasma levels of genistein. These results were the same in pre and post-menopausal women (Verheus et al., 2007).

A number of larger prospective studies (den Tonkelaar et al., 2001; Hirayama, 1992; Horn-Ross et al., 2002; Keinan-Boker et al., 2004; Key et al., 1999; Nomura et al., 1978; Yamamoto et al., 2003) have also been conducted using dietary assessments of intake as a marker for isoflavone status. Only one found significant results (Yamamoto et al., 2003), with consumption of miso soup and isoflavones (not soy foods) inversely associated with risk of breast cancer in Japanese women aged 40-59 years. Compared with those in the lowest quartile of isoflavone intake, the adjusted RRs for breast cancer for women in the second, third, and highest quartiles were 0.76 (95% CI = 0.47 to 1.2), 0.90 (95% CI = 0.56 to 1.5), and 0.46 (95% CI = 0.25 to 0.84), respectively (P for trend =

.043). This association was stronger in post-menopausal women (P for trend = .006) (Yamamoto et al., 2003).

Prospective studies that have examined plasma and urine concentrations of isoflavones include a study in pre-menopausal German women, which found a non-significant positive association between isoflavones and breast cancer risk (Piller et al., 2006) and a study in Dutch postmenopausal women which found a weak but non-significant negative association between genistein urine concentrations and breast cancer risk (den Tonkelaar et al., 2001).

More recently meta-analyses have been conducted to assess soy intake and breast cancer risk. (Trock et al., 2006) analysed 12 case control and 6 cohort studies taking original measures of soy (soyfood intake or urinary isoflavone excretion) and converting them to an estimate of grams of soy protein consumed daily. They found high soy intake was modestly associated with reduced breast cancer risk [OR =0.86, 95% CI =0.75-0.99] and this association was stronger in pre than post-menopausal women [OR=0.7, 95% CI= 0.58-0.85] vs [OR=0.77, 95% CI=0.6-0.98]. (Qin et al., 2006) looked at 14 case-control and 7 cohort studies, including studies published in Japanese and Chinese. They also found an inverse association with soy food/isoflavone intake and breast cancer [RR=0.75, 95% CI=0.59-0.95], with isoflavone intake resulting in a 20% decrease in risk [RR-0.81, 95% CI=0.67-0.99].

1.2.3.2 Hormone levels and breast cancer risk

Reviews of epidemiological and clinical data confirm that lifetime exposure to increasing concentrations of oestrogens and androgens significantly increases breast cancer risk (Clemons & Goss, 2001; Key, 1999; Peeters et al., 2003; Thomas et al., 1997). A review of data from prospective studies has found that levels of endogenous sex hormones are strongly associated with breast cancer

risk in post-menopausal women (Key et al., 2002), whilst a recent case control study nested within the European Prospective Investigation into Cancer and Nutrition cohort, found that elevated blood concentrations of androgens were associated with an increased risk of breast cancer from 370 pre-menopausal women who subsequently developed breast cancer vs 726 matched cancer free control subjects (Kaaks et al., 2005).

Breast cancer patients have been shown to have shorter menstrual cycle lengths than control subjects (Olsson et al., 1983), while Asian women have longer cycle lengths than those in Western populations (Treloar et al., 1967). Women with shorter cycles will have a greater lifetime exposure to oestrogen, and also spend more of their reproductive lifetime in the luteal phase, where the mitotic rate for breast tissue is almost fourfold greater than during the follicular phase. Isoflavone supplementation, by increasing the length of the follicular phase, may therefore reduce the risk of breast cancer. Thus soy isoflavones may reduce risk of breast cancer by changing levels of endogenous hormones and altering the menstrual cycle (Adlercreutz, 2002a) (Bingham et al., 1998).

1.2.4 Intervention studies examining effect of isoflavone supplementation on hormone levels

A number of studies have looked at the effect of isoflavone supplementation on hormonal endpoints, in both pre and post-menopausal women, with varying results, and these are summarised in Tables 1.2.4.1 and 1.2.4.2.

In the controlled environment of a metabolic unit, studies have shown that consumption of isoflavone-rich soy extends the length of the follicular phase and therefore overall menstrual cycle and decreases serum follicle-stimulating hormone and luteinizing hormone concentrations (Cassidy et al., 1995; Cassidy

et al., 1994). However, 36 oz of soy milk had no effect on LH or FSH levels in 10 pre-menopausal women with no change in mean menstrual cycle length (Lu et al., 2000a; Lu et al., 2001). Only two studies have shown a significant reduction in oestradiol concentrations (Lu et al., 1996a; Wu et al., 2000). All of the studies reviewed (Table 1.2.4.1) showed a reduction in total progesterone concentrations measured in the luteal phase but only (Lu et al., 1996a) found this to be a significant reduction. SHBG levels measured in these intervention studies also varied with only two studies showing increases, and they were in the follicular (Nagata et al., 1998) and luteal phase respectively (Wu et al., 2000). In a more recent randomised intervention study with 220 pre-menopausal women (Maskarinec et al., 2004), there appeared to be no significant difference in the oestrone, oestradiol, progesterone and SHBG levels of the intervention group consuming ~50mg isoflavones daily vs control group in samples taken in the luteal phase (day 19) after 3, 6,12 and 24 months. The authors concluded that the effects of soy on the breast, if they exist, are mediated by mechanisms other than the lowering of circulating estrogen levels in pre-menopausal women.

In post-menopausal women Intervention studies have also produced mixed results (Table 1.2.4.2). In post-menopausal women most soy intervention studies reviewed have produced no difference in levels of oestradiol, LH, or FSH (Baird et al., 1995; Berrino et al., 2001; Brooks et al., 2004; Brzezinski, 1997; Duncan et al., 1999b; Foth & Nawroth, 2003; Murkies et al., 1995; Nicholls et al., 2002; Persky et al., 2002; Petrakis et al., 1996; Pino et al., 2000). In the large EPIC Oxford cross-sectional study (Verkasalo et al., 2001), there were no differences seen in oestradiol or SHBG levels of 456 postmenopausal women consuming daily soy milk. Another centre in the EPIC study (Norfolk)

found that urinary and serum levels of daidzein and genistein were negatively correlated with plasma oestradiol (Low et al., 2005) but that when women with certain genotypes were excluded these correlations were no longer significant. The author concluded that polymorphisms in genes encoding for enzymes involved in oestradiol metabolism (i.e. ESR1 PvuII CC genotype) may affect the concentration of plasma oestradiol, suggesting diet-gene interactions. Recent cross-sectional studies of post-menopausal women (Shoff et al., 1998), (Thomas et al., 1999),(Verkasalo et al., 2001), (Low et al., 2005) assessing phytoestrogen intakes through dietary questionnaires found correlations with SHBG and oestradiol levels were not significant.

Results from a number of human studies suggest that soy consumption may exert oestrogenic effects on breast tissue and highlight the fact that caution is necessary at this stage. In a 6-month study (Petrakis et al., 1996), breast fluid secretion in both pre- and post-menopausal women in response to soy protein isolate consumption (containing 37.4 mg genistein/day) increased 2-6 fold, as did the number of atypical cells in breast fluid. However, this was a pilot study, which did not include a control group.

In another study, this time in pre-menopausal women, (McMichael-Phillips et al., 1998), the rate of DNA synthesis by breast cells taken from biopsies of normal breast tissue from women with benign or malignant disease was enhanced by 14 days of soy feeding (60 g/day equivalent to 45 mg isoflavones) prior to surgery. (Hargreaves et al., 1999) also found nipple aspirate levels of apolipoprotein D were significantly lowered and pS2 levels raised in response to soy supplementation ($P < \text{or} = 0.002$), after two weeks of soy supplementation, which they suggest is indicative of an oestrogenic stimulus. However, in a more recent pilot study with 17 surgical patients, after two week isoflavone

supplementation, there appeared to be a statistically non-significant trend towards cancer growth inhibition in the isoflavone treatment group, as manifested by higher apoptosis/mitosis ratios in tumour biopsies compared with those from the control untreated group (Sartippour et al., 2004).

1.3 Insulin like Growth Factor -1 and binding proteins

Insulin like growth factor –1 (IGF-1) originally termed somatomedin (Daughaday et al., 1972) (Van Wyk et al., 1980) is structurally homologous to the hormone insulin and, like insulin, is involved in stimulating the anabolic processess needed for growth during puberty and adult life (Juul, 2003; Ranke et al., 1999). Much of the IGF-1 found in plasma is hepatically produced upon the stimulation of growth hormone released by the pituitary. Like the growth factor itself, the receptors for insulin and IGF-1 are structurally very similar, creating the potential for cross affinity and binding. Indeed, many tissue types, including those of the ovaries and breast, express receptors for both growth hormones. Target tissues also have the capacity to produce IGF-1 locally when needed (D'Ercole et al., 1980) as it is invoved in the paracrine control of the tissue's cellular proliferation. This includes normal breast tissue (Paik, 1992), where it is required for mammary development (Ruan & Kleinberg, 1999). Expression of IGF-1 in recent studies assessed by RT-PCR, show that levels tend to be higher in non-neoplastic breast tissue than in adjacent cancerous tissue (Voskuil et al., 2004) (Chong et al., 2006b) (Chong et al., 2006a) providing evidence for a paracrine relationship between IGF-1 producing stromal fibroblasts and neighbouring epithelial cells (Ellis et al., 1994). Concentrations of IGF-1 in plasma are at least a hundred fold greater than that of insulin. However, IGF-1 is bound to a family of six binding proteins (IGFBP-1

to 6) (Baxter et al., 1998), produced by many tissues and found in plasma. Again growth hormone is responsible for the regulation of these binding protiens and is regulated by the nutritional status of the individual. Binding proteins are also produced locally within the target tissues

More than ninety percent of IGF-1 is bound to IGFBP-3 and an associated acid-labile subunit, forming a high molecular weight complex, which is not available to the tissues (Juul, 2003). Clinical studies suggest that individuals with IGFBP-3 levels in the upper range of normal may have decreased risk of breast cancer while *in vitro* and animal studies show that IGFBP-3 can also regulate cell growth and apoptosis through IGF independent mechanisms (Ali et al., 2003). IGFBP-1, on the other hand, binds only a small fraction of circulating IGF-1, with hepatic production regulated through the portal supply of insulin, with insulin inhibiting its production in the liver (Brismar et al., 1994). Thus, regulation of this binding protein is sensitive to food intake and has been shown to correlate inversely with the secretion of insulin (Kaaks & Lukanova, 2001; Lee et al., 1997b).

1.3.1 IGF-1 and breast cancer risk

Next to oestrogen, Insulin-like growth factor-1 is one of the most potent mitogen for breast cancer cell lines *in vitro* (Karey & Sirbasku, 1988). It can rescue cells from cytotoxic chemotherapy through induction of proliferation and inhibition of apoptosis, stimulating both MAPKinase and PI3Kinase pathways (Gooch et al., 1999; Laban et al., 2003).

In Vivo, antibody blockade of the IGF-1 Receptor inhibits growth of human breast cancer cells in mice (Arteaga et al., 1989) while liver specific gene deletion knockout of IGF-1 gene results in mice with reduced circulating IGF-1

levels and reduced rates of cancer growth and metastasis (Yakar et al., 2005). Lower circulating levels of IGF-1 also delay the onset of induced mammary tumours *in vivo* (Wu et al., 2003).

In humans, significantly elevated concentrations of IGF-1 have been found in breast cancer patients when compared with healthy controls (Bruning et al., 1995; Peyrat et al., 1993). Healthy subjects with high IGF-1 concentrations, when controlled for binding proteins, have been shown to have an elevated risk of breast cancer (RR 7.28, highest vs. lowest quintile (Hankinson et al., 1998). High concentrations of IGF-1 in serum have now been associated with an increased risk of breast cancer, in case control studies (Bohlke et al., 1998; Bruning et al., 1995; Del Giudice et al., 1998; Johansson et al., 2004; Li et al., 2001; Peyrat et al., 1993; Yu et al., 2002) and more recently in prospective studies (Allen et al., 2005; Hankinson et al., 1998; Krajcik et al., 2002; Muti et al., 2002; Rinaldi et al., 2005a; Rinaldi et al., 2006; Schernhammer et al., 2005; Toniolo et al., 2000). It is therefore now accepted that elevated levels of IGF-1 are an independent risk factor for pre-menopausal breast cancer, and this has been confirmed by meta-analysis (Renehan et al., 2004) while a review of recent cohort studies show that associations with IGFBP-3 are less clear (Renehan et al., 2006).

The first study to show a difference in IGF-1 between breast cancer cases and controls compared 44 primary breast cancer patients with 92 controls all over the age of 35. This showed significantly higher levels in the patients, with median concentration of IGF-1 of 152 ng/ml as apposed to 115ng/ml (Peyrat et al., 1993). In a larger study done by (Bruning et al., 1995), 150 Stage I- II breast cancer patients between the ages of 38 to 75 had serum levels analysed for IGF-1 and BP3 just prior to or one month after surgery. These were compared

with 441 women from a population base control group. Pre-menopausal cases showed increased IGF-1, decreased IGFBP-3 and increased IGF-1/BP-3 ratios. The relative risk was 7.34 for the highest compared with the lowest quintile of IGF-1/BP-3.

A more recent study examined levels in 94 patients with pre-menopausal ductal carcinoma-in-situ vs 76 controls and found that women in the two highest tertiles of IGF-1 and the lowest tertile of IGFBP-3 were at 3.7-fold higher risk than women in the lowest tertile of IGF-1 and highest two tertiles of IGFBP-3 (Bohlke et al., 1998).

A prospective study conducted with data obtained in the Nurses Health Study cohort, analysed plasma concentrations of IGF-1 and IGFBP-3 from samples collected in 1989-1990 in women who went on to develop breast cancer after this date up to 1994. They compared these 397 women with 620 aged matched controls. Among pre-menopausal women younger than 50 years, the mean IGF-1 concentration was significantly higher in the cases than in the controls ($p=0.009$) with a relative risk of 4.58 (Hankinson et al., 1998).

Most reviews agree that this relationship is more prominent in pre-menopausal women (Fletcher et al., 2005; Renehan et al., 2004; Schernhammer et al., 2005; Shi et al., 2004; Sugumar et al., 2004) but not all studies have found this (Kaaks et al., 2002; Rinaldi et al., 2006; Rinaldi et al., 2005b), with a recent prospective analysis of the Nurses Health Study II cohort study finding no associations in pre or post-menopausal women (Schernhammer et al., 2006).

The reasons for this discrepancy may be related to the age of participants at recruitment (Rollison et al., 2006) or genetic differences in subpopulations (Al-Zahrani et al., 2006). Polymorphisms of IGF-1 and BP genes have been shown to change risk of breast cancer although authors conclude they do not play a

major role in altering breast cancer risk in Caucasian populations (Canzian et al., 2005) (Canzian et al., 2006).

However the effect of differences in time between blood collection and tumour diagnosis cannot be ruled out given the possibility of tumour influence on IGF levels (Schernhammer et al., 2006). A recent updated meta-analysis of all these studies concludes that there is a modest association between IGF-1 and breast cancer risk in pre-menopausal and young women but no discernible associations between IGF-1, IGFBP-3 and post-menopausal breast cancer risk (Renehan et al., 2006).

1.3.2 IGF-1 and oestradiol

Oestradiol and IGF-1 appear to work together in the proliferation of breast cancer cells with increasing support for cross talking between the ER and IGF-1R signalling pathway (Kato et al., 2000; Laban et al., 2003). Oestradiol has been shown to stimulate expression of IGF-1, the IGF-1R, IRS-1 and down-regulate expression of IGF binding proteins (Huynh et al., 1996b; Lee et al., 1999; Ruan et al., 1995; Stewart et al., 1990), while genistein has been shown to enhance the IGF-1 signalling pathway in proliferation of MCF-7 cells at low concentrations (Chen & Wong, 2004).

1.3.3 IGF-1 and Tamoxifen

The cytotoxic effects of tamoxifen are also partially mediated through IGF-1R signalling (Guvakova & Surmacz, 1997). Tamoxifen has been shown to lower circulating IGF-1 levels by approximately 30% in breast cancer patients (Campbell et al., 2001; Colletti et al., 1989; Helle et al., 1996; Lonning et al.,

1992; Pollak et al., 1990) and healthy women (Bonanni et al., 2001; Decensi et al., 1998; Decensi et al., 2003).

The mechanism by which tamoxifen alters IGF status remains to be fully elucidated. However, tamoxifen can alter IGF-1 levels by reducing the production of GH from the pituitary (Tannenbaum et al., 1992) thereby lowering the amount of IGF-1 released into circulation (Bonanni et al., 2001; Pollak et al., 1990) and in *in vivo* models, directly inhibits the expression of the IGF-I gene in common target organs for breast cancer metastasis (Huynh et al., 1993). It also has direct action as an antiestrogen on breast cancer cells and appears to reduce the number of IGF-1 receptors (Huynh et al., 1996a) and up-regulates expression of binding proteins released by the cells themselves (Huynh et al., 1996b; Winston et al., 1994).

1.3.4 IGF-1 and diet

Although numerous epidemiological and *in vitro* studies positively associate IGF-1 and it binding proteins with increased cancer risk (Renehan et al., 2004) the major determinants of inter-individual variability in circulating IGF-1 levels are not yet fully understood. Studies show that genetic (Fletcher et al., 2005) and nutritional factors, such as energy and protein restriction, play an important role in the regulation of IGF-1 (Thissen et al., 1994).

Reports examining the IGF and diet relationship have mostly been observational and cross sectional human studies are summarised in table 1.3.4.1. These studies have reported associations between IGF-1, BP-3 and BP-1 levels and a range of dietary variables including calcium/dairy foods, fibre, vegetables, fat, carbohydrates and micronutrients, and are discussed in further detail in chapter 5.

1.3.5 IGF-1 and isoflavone/soy intake

A few of the more recent epidemiological studies have also examined the association between IGF-1 and intake of soy/phytoestrogens (Nagata et al., 2003; Probst-Hensch et al., 2003; Vrieling et al., 2004). No correlations were seen between soy product intake and IGF-1 or BP-3 levels in 261 Japanese pre-menopausal women (Nagata et al., 2003) or 386 pre and post-menopausal Dutch women (Vrieling et al., 2004) assessed using food frequency questionaires. However there was a positive association found between soy intake and IGF-1 in 321 Chinese men studied (Probst-Hensch et al., 2003) and a positive association with plant lignans and BP-1 concentrations in 162 postmenopausal Dutch women (Vrieling et al., 2004).

Prior to reports on the cross-sectional associations between isoflavone intake and IGF, two intervention studies were conducted between 1997-9, the results of which are presented in this thesis. These have now been published (Woodside et al., 2006) (Campbell et al., 2004). Since conducting these isoflavone supplementation studies, other intervention studies have also published their findings on the effects of soy or isoflavone supplementation on IGF-1 and binding proteins (these are summarised inTable 1.3.5.1). Of the studies published to date, only our study (Campbell et al., 2004) and to our knowledge one subsequent study published very recently (Vrieling et al., 2007) have looked at the effects of isoflavone supplementation alone. Further discussion of these intervention studies can be found in chapter 5.

1.4 Conclusion

In vitro and animal evidence suggests that dietary isoflavones may be protective against the development of breast cancer. Epidemiologic evidence remains

largely inconclusive, and while case-control studies have produced some convincing evidence that increased dietary isoflavone intake correlates with reduction in breast cancer risk, early intervention studies have produced conflicting results. In light of the emerging role for IGF-1 and its binding proteins in breast cancer aetiology, this thesis aims to examine the effects of Isoflavones on IGFs, to determine whether, like other antiestrogens, this may be a possible mechanism of action, which could further explain the possible protective effects of these compounds in the diet.

We propose to do this by first examing the effects of the SERM tamoxifen on levels of IGF-1 and its binding proteins in breast cancer patients, then by looking at the short-term effects (single dose or one week supplementation) of a phytoestrogen-rich bar and then a placebo-controlled isoflavone supplementation study over one month on IGF-1 and binding proteins, in healthy pre and post-menopausal women. Finally we examine the effects of isoflavones on DNA synthesis in breast cancer cell lines *in vitro* to compare their actions with tamoxifen in the presence or absence of IGF-1 and oestradiol.

Table 1.2.3.1. Studies on the association between phyto-oestrogens in plasma and urine or phyto-oestrogen-rich diet and breast cancer risk.

Table 1.2.4.1: Interventions with soy foods/supplements in premenopausal women-effects on hormones

Table adapted from (Maskarinec et al., 2004)

Table 1.2.4.2: Interventions with soy foods/supplements in postmenopausal women- effects on hormones.

Author	Isoflavone dose; type of food	Length (mo)	Type	n	E1 (%)	E2 (%)	SHBG (%)	LH (%)	FSH (%)
Baird et al. 1995	165mg; soy foods	1	RI RC	66 25	ND	ND	-6.8 -5.7	-2.5 -2.0	-4.4 -2.8
Murkies et al. 1995	45g soy or wheat flour	3	RI (soy) RC (wheat)	23 24	ND	ND	ND	ND	-1.6 -9.8**
Petrakis et al. 1996	75 mg; soy protein isolate	6	CO	10	ND	-0.94	+44.4	ND	ND
Brzezinski et al. 1997	~375 mg; tofu, soy drink, miso and flax seed	3	RI RC	78 36	ND	-22.2 -13.6	+27.6* -7.5	ND	ND
Duncan et al.1999	10, 65, 129 mg; soy protein isolate	3	CO (10vs129)	18	-5.6	-11.9	+5.6*	+5.5	+3.04
Knight et al. 1999	40 or 160 mg	3	RI (40) (160)	37	ND	ND	-11.7 -16.3	ND	-6.3 -16
Pino et al. 2000	69mg; soy milk	10wks	Interven	20	-8.59	+0.17	+15.7*	ND	-10.2
Berrino et al. 2001	~45 mg; fibre and phtoestrogen rich diet	5	RI RC	49 50	ND	-18.0 -5.5	+25.2* +3.6	ND	ND
Persky et al. 2002	56, 90 mg; isolated soy protein	6	RI RI RC	24 23 25	ND	+40 +10.2 -18.6	-12.8 -5.2 -17.2	ND	-7.3 -0.68 -1.4
Foth et al. 2003	20mg; soy protein	6	Interven	16	ND	-15.2	ND	+3.6	+2.2
Brooks et al. 2004	41.9mg; soy flour in muffin	4	RI RC	16 15	+40 +5.25	+89.6 -24.7	ND	ND	ND
Nicholls et al. 2002	60mg; textured soy protein	14 days	Interven	7	ND	+25.6	ND	-6.9	-3.3
Arjamandi et al. 2005	60mg; 25g protein from soy products or control protein with no soy or isoflavones.	12	RI[b] RC[b]	35 27	+14.3 -2.8	+39.4 -11.1	-14.5* -12.4	ND	-3.6 -11.2

Table 1.3.4.1: Epidemiological studies- Dietary correlations with IGF-1, BP3 and BP1 concentrations

Author	n	Subjects	Results			
			IGF-1	IGFBP-3	IGF-BP-1	other
Devine et al 1998	119	Postmenopausal women	Zinc +ve Protein +ve	Riboflavin +ve	ND	Baseline-prior to controlling for age and weight, IGF-1 correlated with Calcium, Cholesterol, Energy, Iron, potassium, magnesium, sodium, niacin, phosphorous, protein, riboflavin, thiamine and zinc.
Kaklamani et al. 1999	115	73%female and 27% male; age 30-84	Red meat +ve Fat +ve Oils +ve Energy from lipids +ve Energy from carbo +ve	Saturated fat -ve	ND	
Ma et al. 2001	193 vs 318	Men, Cases with colorectal cancer vs controls; age 40-84 (physicians health study)	Dairy food +ve	Dairy food +ve	ND	
Mucci et al 2001	112	Men, control group for liver cancer study. Greek population; all ages.	Alcohol –ve Cooked tomatoes -ve	Not shown	ND	IGF1/BP3 ratio: Cooked tomatoes -ve
Allen et al 2002	292	Women, age 20-70, Meat eaters vs vegetarians vs vegans	Animal protein +ve Animal plus soy protein +ve Nonsoya plant protein –ve NSP -ve	None	Nonsoya plant protein +ve SFA –ve NSP +ve Zinc +ve	IGF1 13% lower in vegan woen than Meat eaters of vegetarians (p=0.006). BP1 20-40% higher in vegans than ME and Vegans(p=0.005). Significant increase in IGF-1 in vegan women who drink ≥ ¾ pint of soya milk daily.
Holmes et al 2002	1037	Women, age 43-69, 526 pre & 456 postmenopausal	B-cryptoxantine +ve Animal protein +ve	Alcohol +ve Sat fat intake –ve Lycopene +ve Vitamin C +ve	ND	Cereal, pasta, milk and total dairy +ve associated with IGF-1

			Vitamin D +ve Vitamin A +ve Calcium +ve Zinc +ve Energy +ve (with BMI <25)	Energy +ve		
Baibas et al 2003	620	225 men & 395 women, age 35-65, Greek EPIC study	Ethanol –ve Saturated lipids +ve	Not showen	ND	
DeLellis et al 2004	1000	Multiethinc; age 45-75; 50% male/female	Men: fat from meat –ve Low fat milk -ve	Men: total fat –ve Saturated fat –ve Fat from meat – ve Low fat milk -ve Women: alcohol +ve	ND	
Giovanucci et al 2003	753	Men; age 40-75; health professionals follow up study	Animal & vegetable protein +ve (milk, fish & poultry) Potassium +ve Zinc +ve Magnesium +ve Calcium +ve Phosphorous +ve Energy intake +ve(men with BMI <25)	Not shown	ND	IGF1/BP3 ratio: Vitamin E Phosphorous had the strongest association with IGF-1 probably because a good marker of both meat and dairy intake.
Gunnell et al 2003	344	Men; age 50-70	PUFA +ve Calcium +ve Dairy products +ve	PUFA +ve	ND	IGF1/BP3 ratio: inversely related to vegetable intake and tomato containing products. Carbohydrate had a +ve trend with IGF-1 levels but not significant.

			Milk +ve			
Heald et al 2003	257	Multiethnic; age 25-78; ~50% men/women	Fat intake +ve Sat fat +ve Protein +ve Carbohydrate +ve Starch +ve Energy +ve	ND	Total fat -ve PUFA -ve Total protein –ve	
Nagata et al 2003*	261	Premenopausal women; age 20-54; Japanese	Vitamin D +ve	Total fat -ve	ND	No correlations seen with soy product intake and IGF-1 or BP-3 levels
Probst-Hensh et al 2003*	338	321 men and 326 postmenopausal women; age >50; Singapore chinese	Soy +ve (men) Calcium +ve Cholesterol +ve LDL +ve Triacylglycerol +ve Total Chol/HDL +ve	Sat Fat –ve Omega 3 PUFA +ve Fibre +ve Cholesterol +ve LDL +ve Triacylglycerol +ve Total Chol/HDL +ve	ND	Inverse trend with Alcohol intake and IGF-1 levels
Vrieling et al 2004*	286	224 pre & 162 postmenopausal women; age 49-69; EPIC study in Netherlands	Alcohol –ve (premen)	Alcohol +ve (premen)	Postmen: alcohol –ve Plant lignans +ve Cereals -ve	No correlations seen with soy intake/phytoestrogens and IGF-1 or BP-3 levels
Larsson et al 2005	226	Men; age 42-76; Sweden	Protein +ve Zinc +ve Red meat +ve Potassium +ve	ND	ND	Fish & seafood +ve trend with IGF-1
Maskarinec et al 2005	258	Premenopausal women; age 34-46; multiethnic	Dietary fibre +ve Vitamin A +ve	Zinc +ve	ND	IGF1/BP3 ratio: Iron +ve Calcium +ve Saturated fat +ve trend. No ethinc differences.

Norat et al 2006	2109	Women; age 35-70; EPIC study in Europe	Protein +ve Calcium +ve Magnesium +ve Phosphorus +ve Potassium +ve Vit B6 +ve Vit B12 +ve Beta- carotene-ve	Calcium +ve Phosphorus +ve	ND	Adjustment of IGF-1 for BP3; PUFA –ve Zinc not analysed as could not standardise estimates across countries. Food correlations with IGF-1 were Milk +ve, Cheese +ve and Vegetables -ve

Table 1.3.5.1: Interventions with soy foods or supplements – effects on insulin like growth factors and binding proteins

Author	Isoflavone dose; type of food	Length (mo)	Type	n	Results %change IGF-1	IGFBP-3	IGF-BP-1	other
Maskarinec et al 2005	50mg;soy foods	24	RI RC	97[a] 99[a]	+0.48 +0.26	-1.47 -0.33	ND	Iso excretion +ve assoc with IGF-1 (p=0.04)
Wangen et al. 2000	10, 65,129mg soy protein isolate	3	CO	14[a] 17[b]	+10.8(PO)* 65 -6.54 (129)*	+5(EF)65 -4.5(129)	ND ND	
Adams et al. 2003	3 vs 83mg; soy protein	12	RI (3) RC (83)	58[bc] 65[bc]	+8.1* +7.7*	0 +1.0	ND ND	
Khalil et al. 2002	88mg; soy protein vs milk protein control	3	RI[c] RC[c]	24 22	Inc* Inc (figures not given)	ND	ND	N.B Only Soy protein supp significantly increased IGF-1 from baseline (<0.0001)
Kaaks et al. 2003	~45 mg; fibre and phytoestrogen rich diet	5	RI[b] RC[b]	50 49	-5.9 -4.4	-4.5 -2.2	+12.2* -6.4	
Campbell et al. 2004	86mg; isoflavone supplement (no protein) & placebo	1	CO	16[a] 7[b]	+1.5 (s Vs P) +2.77	+2.5 -1.1	+13.2 +12.3	
Woodside & Campbell et al. 2006	80mg; soy and linseed bar (18g protein)	1 week	interven	10[ab]	+71*	18.3*	-40.7	
Arjamandi et al. 2003	88mg; soy protein vs milk protein control	3	RI[b] RC[b]	20 22	+68.6** +36.4**	ND	ND	Increases in IGF-1 greater for women on HRT.
Arjmandi et al. 2005	60mg; 25g protein from soy products or control protein with no soy or isoflavones.	12	RI[b] RC[b]	35 27	+26.3** +12.8**	+1.85 +5.2**	ND	Both significantly increased IGF-1 but IGFBP-3 only sig increased on soy.
Spentoz et al. 2003	114mg; soy protein supp on low fat diet	2	Interven[c]	15	+22	-1	ND	Increase in IGF-1 of 9% after low fat diet then increase by 22 % from baseline on

								soy protein p=0.02
Gann et al 2005	88mg; soy protein powder with or without Isoflavones (subjects on both LFHF and UD)	3	RI[a] RC[a]	153	2.9 1.2	-1.2 -3.4*	7.5 6.3	Small but significant decrease in BP3 of 2.3% when both groups analysed together.
Vrieling et al 2007	84mg; isoflavone supplement (no protein) & placebo	2	CO	37[c]	-2.5	+0.43	-6.4	Change in IGF-1 and serum Equol were negatively correlated post supplement.

a =premenopausal
b=postmenopausal
c=men

CHAPTER TWO – METHODS

2.1 Collection and preparation of biological samples

Urine samples (24 h) were stabilised with 1-2 g/l ascorbic acid and sodium azide (0.1 g/L) and stored at -80°C until analysis. Spot urine samples were not stabilised but immediately frozen after collection. Blood samples either kept at 4°C for a maximum of 30 min (plasma) or were allowed to clot for 30 minutes in the dark (serum) and then centrifuged at 3000 rpm for 10 min. Serum or plasma was aliquoted and stored at -80°C until analysis. Lymphocytes were collected into a Vacutainer Cell Preparation Tube (Becton Dickinson, New Jersey), extracted and washed according to manufacturer's instructions and stored at -80°C.

2.2 Endpoint measurements

2.2.1 Diet

Food frequency questionnaires (from EPIC) and food diaries were analysed using DietPlan 5 for Windows 98 using standardised composition (McCance et al., 1978) and food portions established by MAAF (Crawley et al., 1988) to assess background diet through out the studies.

2.2.2 Phytoestrogens

Urine samples were analysed for the isoflavonoids genistein and daidzein, the isoflavan equol and the lignans enterolactone and enterodiol levels, using gas chromatography-mass spectrometry following enzyme hydrolysis, extraction into ether and silylation according to Morton et al. (Morton et al., 1994).

2.2.3 Ascorbic acid

Ascorbic acid concentrations were determined as described by Vuillemier & Keck (1989) (Vuillemier & Keck, 1989). The procedure is based on the enzymatic oxidation of ascorbic acid and subsequent quinoxaline formation to generate a fluorescent derivative measured on the Cobas Fara centrifugal analyser. The whole blood sample, stabilised with dithiothreitol (Fisher Scientific), was centrifuged and separated upon arrival at the laboratory. The plasma was precipitated by adding 100 μl of plasma to 900 μl of 5% metaphosphoric acid (Fisher Scientific) in a conical microcentrifuge tube and vortex mixed. Once precipitated the sample was stable for 1 year at -70°C. Our samples were assayed within 6 months of collection. Working standards (0, 1, 5, 10, 15, 20 μmol/l) were prepared for each run from a freshly made stock standard of 20 μmol/l ascorbic acid in 5% metaphosphoric acid. Approximately 200 μl of standards and samples were placed in Cobas cups, and oxidising reagent (9.9 ml, 2 mol/l acetate buffer and stock ascorbate oxidase) and coupling reagent (20 mg of 1,2-phenylenediamine dissolved in 20 ml deionised water) added to appropriate reagent wells. The assay was then started. The intra-assay CV was calculated for several different serum samples. At a mean concentration of 4.2 μmol/l, the inter-assay CV was 6.80% (n=9), at 50.6 μmol/l the inter-assay CV was 0.72%, while at 151.0 μmol/l the inter-assay CV was 1.50%. Antioxidant malondialdehyde (MDA) levels in serum were analysed using HPLC method (Young & Trimble, 1991).

2.2.4 Retinol, α-tocopherol and carotenoids

Concentrations of serum α-tocopherol and β-carotene were measured by HPLC according to the method of Catignani *et al.* (1983)(Catignani & Bieri, 1983) and Thurnham *et al.* (1988) (Thurnham et al., 1988). They were monitored by ultraviolet detection following extraction into heptane. The assay also measures serum concentrations of retinol, lycopene and α-carotene. Serum or standard (200 μl) was pipetted into 4 ml glass tubes. Internal standard, a mixture of retinol acetate and α-tocopherol acetate in ethanol (400 μl - Labscan) was added to each tube and the ethanol precipitated the protein from the serum. Heptane (Analar or 99% - 700 μl (Labscan)) containing 0.5 g/l butylated hydroxytoluene (BHT) was added to the tubes which were then vortexed vigorously for 1 minute. The tubes were then centrifuged for 10 minutes at 1200 g and 500 μl of the resulting top heptane layer transferred to a set of identically labelled glass tubes. The complete contents (1300 μl) of the standard tube contents were dried. These tubes were evaporated to dryness in a rotary evaporator under vacuum for 1- 2 hours. The samples were either assayed immediately or stoppered and stored dry in a -70°C freezer. For HPLC analysis, the samples were reconstituted in ethanol (100 μl), vortexes and assayed using UV detection. A 45% methanol:45% acetonitrile:10% dichloromethane (200 ml, not filtered, degassed for exactly 5 minutes using Romil HPLC grade solvents, (Analab, Dromore, Co. Down) mobile phase was used with a flow rate of 1.8 ml/min. The column used was Hypersil ODS 5 μm (Alltech), with changing wavelengths of 325 nm, 292 nm and 450 nm to pick up the retinol, tocopherol and carotenoid peaks respectively. A Thermo Separation Products Constametric 4100 pump was coupled with a Spectra-Physics AS1000 autosampler and a Spectra-Physics Analytical UV2000 ultraviolet

detector. The system was controlled using a Spectra-System SN4000 interface and operated using PC1000 software from Thermo Separation Products.
A quality control serum sample was assayed at the start of every batch of samples. The inter-assay CV for α-tocopherol was 5.4% (n=7) and 10.0% (n=7) for β-carotene. The CVs for retinol, lycopene and α-carotene were similar to those for β-carotene. The addition of internal standard allowed the calculation of an intra-assay CV of 4.9% (n=29).

2.2.5 Serum Lipids and lipid peroxidation

Serum lipids were assessed, again at the start and end of each supplementation phase, using standard spectrophotometric assays.
Serum total cholesterol, was estimated using an enzymatic CHOD-PAP kit, while serum triglycerides were measured using the Peridochrom GPO-PAP kit (both Boehringer Mannheim). Precipitation for HDL-cholesterol estimation employed phosphotungstic Mg^{2+} reagents (Lopes-Virella *et al.*, 1977). All cholesterol assays were carried out on the Cobas Fara auto-analyser. As well as employing internal quality control procedures, total cholesterol and HDL-cholesterol were subject to external quality control through the World Health Organisation reference laboratory in Prague. LDL-cholesterol was estimated using the Friedewald formula:

LDL cholesterol (mmol/l) = total cholesterol - HDL cholesterol - 0.45 × triglyceride

Levels of aqueous phase lipid hydroperoxides were assessed in plasma by the FOX 1 (ferrous oxidation in xylenol orange) assay according to Wolff (Wolff,

1994). Susceptibility of LDL to oxidation was assessed according to McDowell et al. (McDowell et al., 1995).

2.2.6 COMET Assay

DNA damage was analysed by the COMET assay in both lymphocytes according to Spanswick et al. (Spanswick et al., 1999).

2.2.7 Creatinine

Creatinine concentrations from 24 hour urine collections were measured using a Sigma colorimetric assay.

2.2.8 Hormones

17 β-oestradiol, Progesterone, LH, FSH and SHBG were measured in serum using routine IRMA/RIA methods as follows:

17 β-oestradiol was measured directly using the DiaSorin Clinical Assays™ Oestradiol-2 Radioimmunoassay. The standards, controls and samples were incubated with oestradiol tracer and antiserum raised in rabbits for 2 hours at 37°C in a water bath. After a second incubation for 15 min with a precipitating reagent, containing antibody to rabit IgG the samples were centrifuged for 15 min at 2000G to separate free tracer from antibody-bound tracer. The tubes were then decanted and the supernatant discarded. The pellet was counted in a gamma counter. A standard curve was then prepared from the standards ranging from 10 to 2000pg/ml. The unknown values were obtained from the standard curve by interpolation.

Progesterone was measured using the DiaSorin Clinical Assays™ GammaCoat™ Progesterone-^{125}I RIA Kit for the quantitative determination of

progesterone levels in serum. This was a competitive binding radioimmunoassay whereby the amount of [^{125}I]-labeled progesterone bound to the antibody on the plastic coated tube was inversely proportional to the concentration of progesterone present in the serum. The samples, standards and controls were added to the Anti-Progesterone pre-coated tubes in duplicate. Progesterone tracer was then added and the tubes were incubated at 37°C for 60 minutes. The tubes were then decanted and counted in a gamma counter. A standard curve was then prepared from the standards ranging from 0.3 to 60ng/ml. The unknown values were obtained from the standard curve by interpolation.

Serum Luteinizing Hormone (LH) and Follicle-stimulating Hormone (FSH) were measured using the NETRIA Immunoradiometric assay (IRMA). The samples, standards and controls were added to the tubes along with the solid phase (sheep) Anti-LH or FSH, the [^{125}]-iodine labelled Anti-LH or FSH (monoclonal) and the assay buffer. These were vortexed and placed on a rotary mixer overnight. The next morning, 2ml of wash buffer were added to the tubes and after centrifugation at 1000G for 5 minutes, the supernatant was decanted. This washing step was repeated before the tubes were decanted and counted in a gamma counter. A standard curve was then prepared for LH and FSH from the standards ranging from 1-260 U/L or 0.5-200 U/L respectively. The unknown values were obtained from the standard curve by interpolation.

Sex Hormone Binding Globulin was measured in serum using the [^{125}I] Immunoradiometric Assay kit from Orion Diagnostica. This procedure was based on the principles of a non-competitive "liquid-phase" immunoradiometric assay. Samples, standards and controls were diluted in assay buffer and added in duplicate to the test tubes. Equal volumes of SHBG Antiserum (Rabbit) and

[^{125}I] anti-SHBG (Mouse monoclonal) were added to all tubes. Tubes were vortexed and incubated at room temperature for 60 minutes. The separation reagent, containing goat anti-rabit IgG covalently bound to solid particles, was then added and tubes were vortexed and incubated for a further 15 minutes at room temperature. Two ml of 0.9% saline was added and the tubes centrifuged at 2000G for 15 minutes. The tubes were decanted and counted in a gamma counter. A standard curve was then prepared from the standards ranging from 6.25, 200 nmol/L. The unknown values were obtained from the standard curve by interpolation.

2.2.9 IGF-1, BP-1 and BP-3

Levels of IGF-1, IGF-BP1, IGF-BP3 were measured in serum using ELISA methods (Diagnostic Systems Laboratories). The DSL-10-5600 IGF-1 ELISA kit was an amplified "one step" sandwich-type immunoassay. This assay included an extraction step in which IGF-1 was separated from its binding protein in serum by acidification followed by ethanol precipitation of the IGFBP fraction. The standards, controls and extracted samples were then incubated with anti IGF-1 antibody labelled with the enzyme horseradish peroxidase (HRP) in microtitration wells coated with another anti-IGF-1 antibody. After incubation and washing, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450nm with background wavelength correction set at 620nm. The absorbance measured was directly proportional to the concentration of IGF-1 present. The IGF-1 standards were used to plot a standard curve of absorbance versus IGF-1 concentration and the IGF-1 concentrations were

then calculated using the line of best fit plotted using the MINITAB statistical package. Controls were assayed at the beginning and end of the plate while samples were assayed in duplicate.

IGFBP-3 and total IGFBP-1 were measured using the DSL-10-6600/7800 ELISA kits. In these "two-step" immunoassays, standards, controls and samples were incubated in microtitration wells which had been coated with an anti-IGFBP-3/1 antibody. After incubation and washing, the wells were treated with another anti-IGF-3/1 antibody labelled with HRP. After a second incubation and washing step, the wells were incubated with TMB. After adding the acidic stopping solution, the enzymatic turnover was measured at 450 and 620nm as above. The absorbance measured was directly proportional to the concentration of BP present. The standards were used to plot a standard curve of absorbance versus BP concentration and the BP concentrations were then calculated using the line of best fit plotted using the MINITAB statistical package.

2.3 Development of an Enzyme Linked Immunosorbant Assay (ELISA) for the quantitative measurement of IGF-BP3 in Human Serum.

Work carried out under supervision of Dr Brian Rafferty at the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

2.3.1 Introduction

Prior to commencing the intervention studies described in this thesis, an attempt was made to develop an in-house assay for IGFBP-3. IGFBP-3 is the major binding protein for IGF-1 in human serum, and the best indicator of clinical conditions involving Growth Hormone deficiencies (Kim et al., 2001) and is also potentially useful as an anti cancer molecule/biomarker (Ali et al., 2003). ELISAs are the preferred method for measuring Insulin Like Growth Factors and their binding proteins (Rutanen & Pekonen, 1991). At the time of this research, ELISA kits were only commercially available from Diagnostics Systems Laboratories (Diamandi et al., 2000). These were expensive, therefore the development of an in-house ELISA would be have been useful. This work would also allow a better theoretical understanding of ELISAs and could hopefully be used as an alternative to commercial kits in future.

2.3.2 Method

An antibody to IGF-BP3 had previously been raised in White Long Eared Rabbits in the Animal House at NIBSC. IGF-BP3 was human recombinant DNA

(non glycosylated) and was from the standardised stock made at NIBSC. Both the Antibody and the Binding protein had been previously assessed using Radio-iodination and Fast Protein Liquid Chromotography (FPLC).

The assay development involved;

- Coating the 96 well microtitre plate with antibody over night (optimal concentration and volume to be determined).
- Coating the Plate with samples and standards (optimal concentration and volume to be determined).
- Addition of Biotinylated Antibody (optimal concentration and volume to be determined).
- Addition of Avidin Horse Radish Peroxidase and Substrate Reagent for colour development (optimal concentration and volume to be determined).
- Incubation (optimal number of steps time and temperature to be determined between each stage).

Assays which attempted to optimise the assay as detailed above are described below in results.

2.3.3 Results

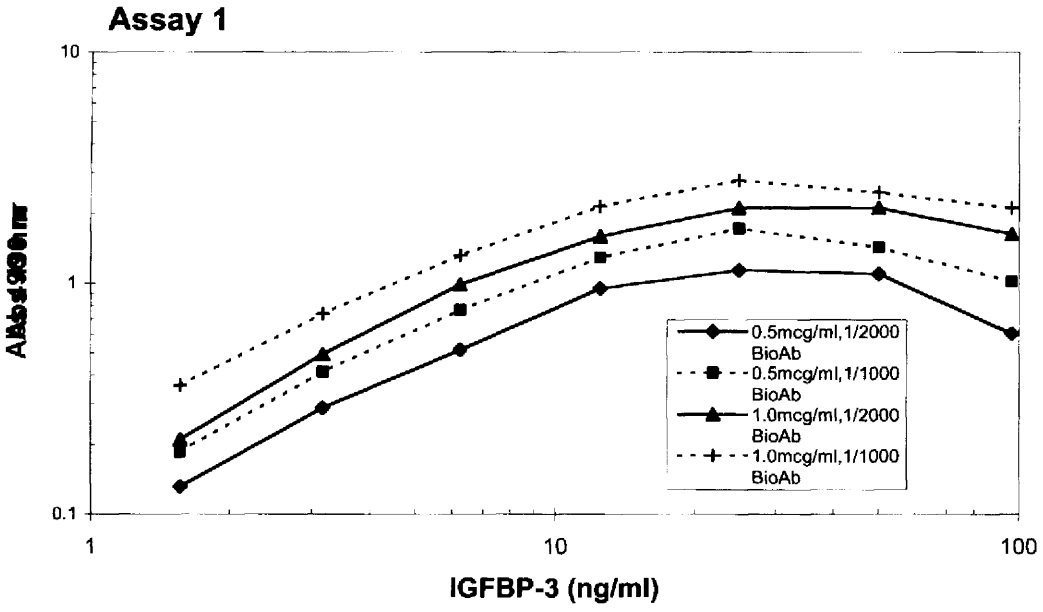
Assay 1:

Aim: To determine the concentration of the coating antibody needed to give adequate binding of the substrate and to establish the dilution of biotinylated antibody needed to give an adequate signal.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	200µl @ 0.5µg/ml 200µl @ 1.0 µg/ml
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	100µl @ 100ng/ml □1.5625ng/ml (2 fold dilns)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/1000 diln 100µL @ 1/2000 diln
INCUBATION STEPS	1 step, 3hr

Results:



Findings: Absorbance readings using 1.0g/ml coating antibody and 1/1000 diln of biotinylated antibody are consistently greater as would be expected.

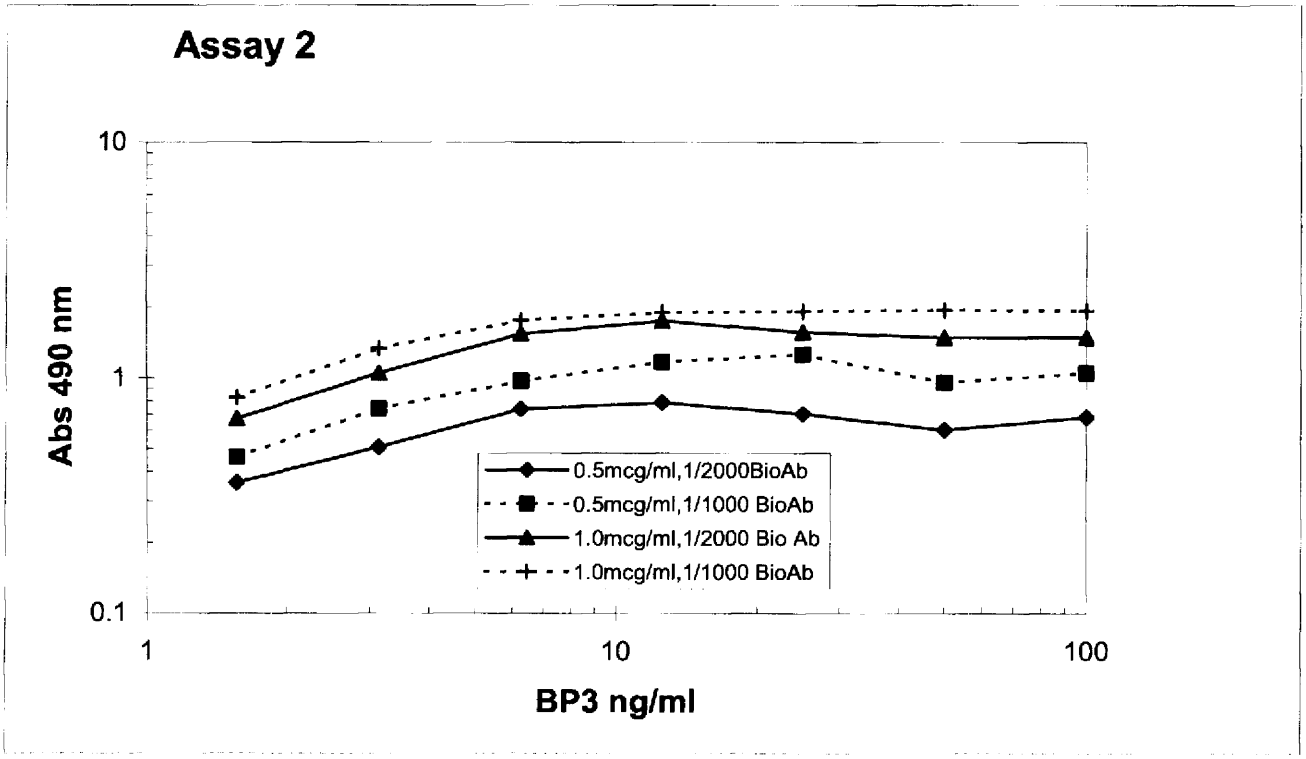
Assay 2 & 3:

Aim: To determine the effect of halving the volumes used in Assay one.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 0.5µG/ML 100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	50µl @ 100ng/ml □ 1.5625 ng/ml (2 fold dilns)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	50µl @ 1/1000 diln 50µl @ 1/2000 diln
INCUBATION STEPS	1 step, 3hrs

Results:



Findings: Halving the volumes used gives an adequate signal if maximal parameters are used. (See assay one) The control (NSB) values were consequently lower in Assay 2.

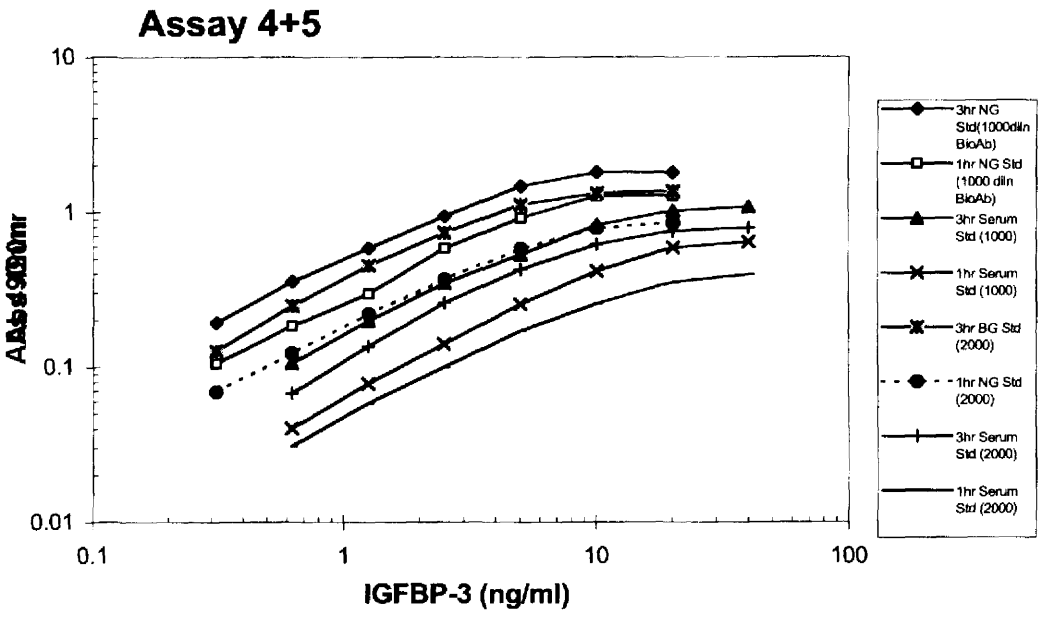
Assay 4 & 5

Aim: To assess the effects of altering incubation times from 3 hours to one.

Method

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0 µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	50µl @ 20ng/ml □ 0.3125ng/ml (2 fold dilns) 50µl @ 40ng/ml □ 0.6125ng/ml (2 fold dilns)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	50µl @ 1/1000 diln 50µl @ 1/2000 diln
INCUBATION STEPS	Assay 4: 1 step, 1 hr Assay 5: 1 step, 3 hr

Results:



Findings: The 3 hour incubation gives an appropriately larger signal while the 1 hour incubation lends itself to a loading effect due to differing incubation with the coating antibody.

Assay 6

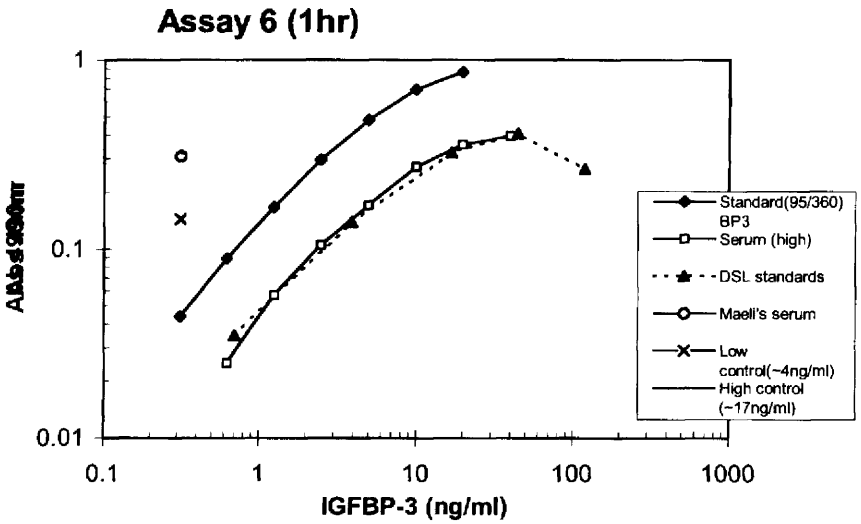
Aim: To assess the DSL standards and control solutions VS the serum pool and NG IGFBP-3 over one hour incubation.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	50µl @ 20ng/ml □ 0.3125ng/ml (2 fold dilns) 50µl @ 40ng/ml □ 0.625ng/ml (2 fold dilns) 50µl @ 0.7, 4, 17, 45, 120ng/ml & 4±1.2, 17±5

	50µl @ 1/50 & 1/100 diln
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	50µl @ 1/1000 diln
INCUBATION STEPS	1 step, 1hr

Results:



Findings: This assay showed quite clearly that the serum responds differently to the NG BP-3 while DSL standards are derived from human serum.

Assay 7

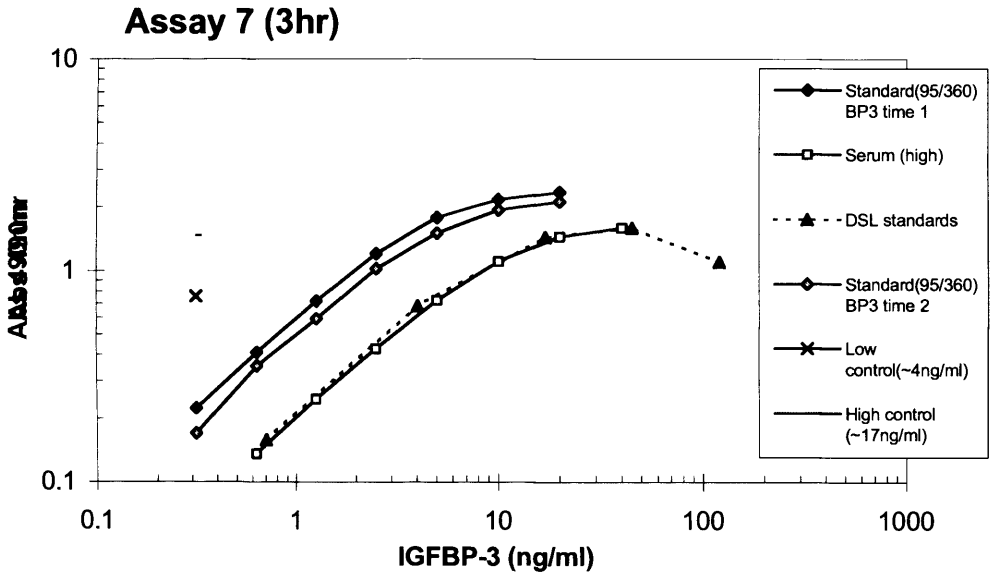
Aim : To assess the effects of a 3 hour incubation on an assay similar to assay 6 and to determine whether time delay in loading a plate will make a significant impact on absorbance readings.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) beginning and end of plate ~ 15 min	50µl @ 20ng/ml □ 0.3125ng/ml (2 fold dilns)

SERUM POOL DSL STD MAELI'S SERUM	50µl @ 40ng/ml □ 0.625ng/ml (2 fold dilns) 50µl @ 0.7, 4, 17, 45, 120ng/ml & 4±1.2, 17±5 50µl @ 1/50 & 1/100 diln
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	50µl @ 1/1000 diln
INCUBATION STEPS	1 step, 3 hr

Results:



Findings: Three hours gives a better signal but a plate loading effect is still visible.

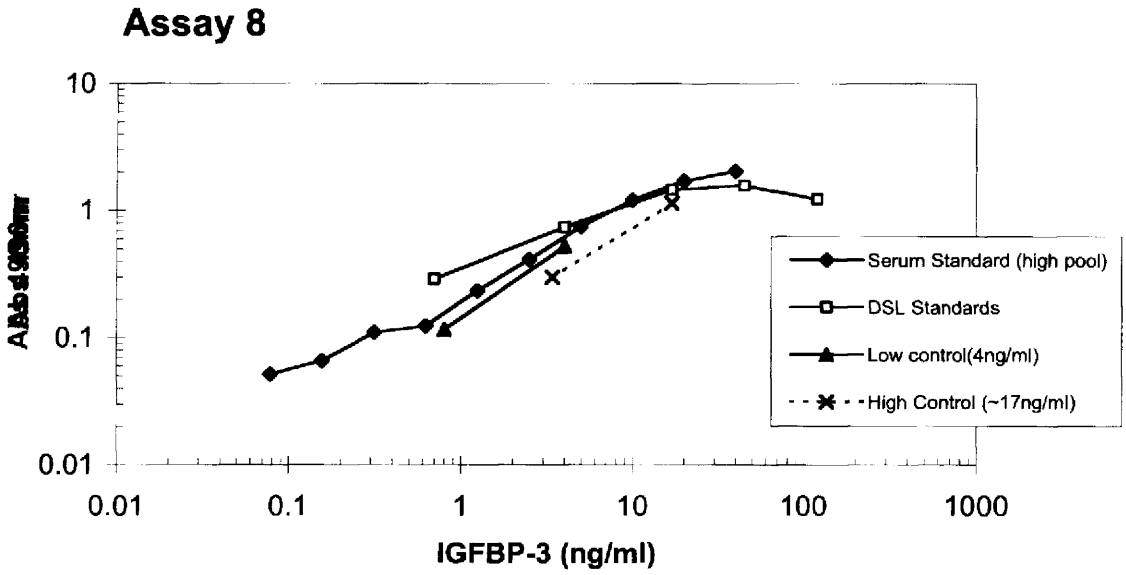
Assay 8

Aim: To extend the sensitivity of the assay while using the serum pool as the standard.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0 µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	 50µl @ 40µg/ml(1/100) □ 1/25600 0.7, 4, 17, 45, 120ng/ml % 4±1.2, 17 ±5 (x 4)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/1000 diln
INCUBATION STEPS	1 step, 3 hr

Results:



Findings: This assay appeared to have an effect across the two halves of the plate, however these results are not.

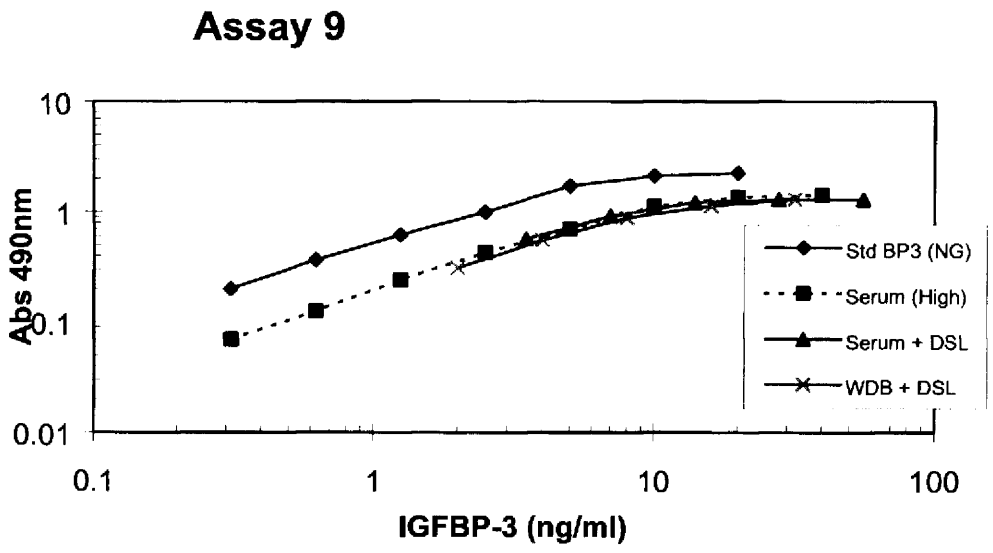
Assay 9

Aim: To assess the effects of spiking the serum pool with known standards to establish recovery values.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD	
NG IGFBP-3 (93/560) (3.5µg/ml)	50µl @ 20ng/ml → 0.312ng/ml (2 fold dilns)
SERUM POOL DSL STD	50µl @ 40ng/ml → 0.3125ng/ml (2 fold dilns)
MAELI'S SERUM	Serum + DSL 50µl @ 56ng/ml→3.5ng/ml (2 fold) Serum + WDB 50µl @ 32ng/ml→2.0ng/ml(2 fold)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	50µl @ 1/1000 diln
INCUBATION STEPS	1 step, 3 hrs

Results:



Findings: The spiked dilution's appear to give readings as would be expected. These need to be extended.

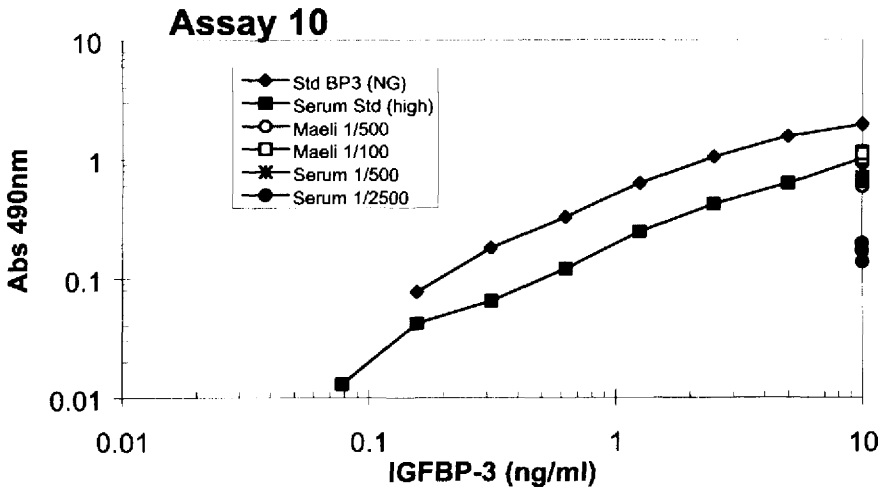
Assay 10

Aim: To assess the sensitivity of the assay by extending the lower end of the standard serum curve. To determine the IGFBP-3 levels in an 'unknown' serum.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	 50µl @ 10ng/ml → 0.156ng/ml (2 fold dilns) 50µl @ 10ng/ml → 0.078ng/ml (2 fold dilns) 50µl @ 1/500, 1/2500 diln 50µl @ 1/100, 1/50 diln
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	50µl @ 1/1000 diln
INCUBATION STEPS	1 step, 3 hrs

Results:



Findings: Providing enough dilutions are done for the unknown serum the standard curve appears to give consistent results. Further work needs to be done to further test this.

Assay 11 & 12

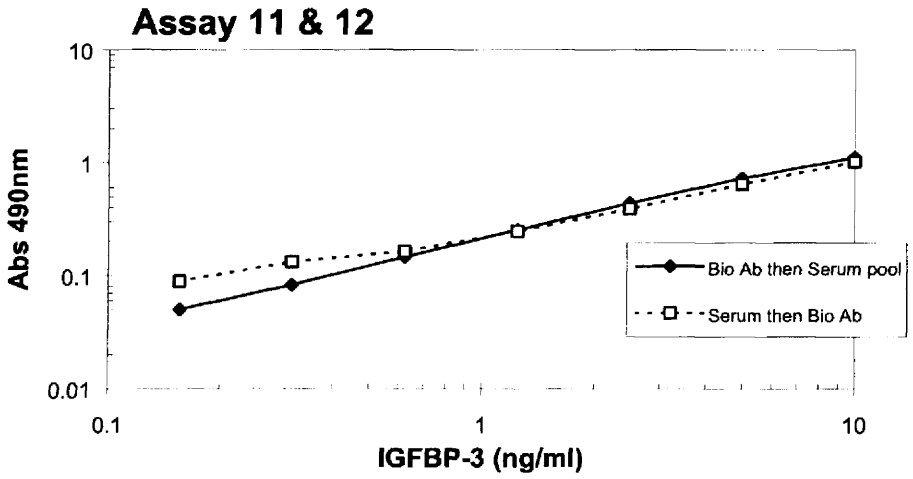
Aim: To determine whether there is a difference in signal when the biotinylated antibody is loaded on to the plate before the serum in order to diminish any plate effect obtained through difference in binding times. (see assay 7)

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	50µl @ 10ng/ml → 0.15625ng/ml (2 fold dilns)(x2 on Assay 11 + 12)

BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	Assay 11: 50µl @ 1/1000 before serum Assay 12: 50µl @ 1/1000 after serum
INCUBATION STEPS	1 step, 3hrs

Results:



Findings: The results would suggest that although there is no great difference between the curves, the control (NSB) values for assay 12 are significantly lower.

Assay 13

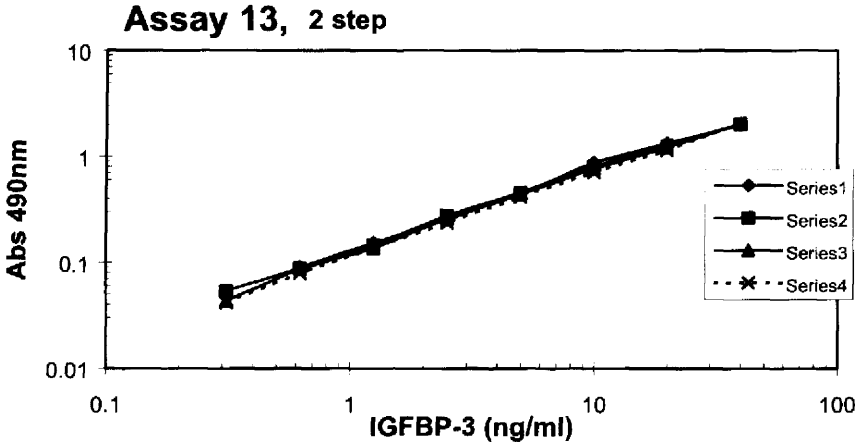
Aim: To assess the affects of a two step incubation on the overall sensitivity and loading effects of the assay.

Methods:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML

SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	100µl @ 40ng/ml → 0.3125ng/ml (2 fold dilns) (x4)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/1000 diln
INCUBATION STEPS	2 step: 2 hrs then 1 hr

Results:



Findings: Although this assay appears to have remedied the plateau effect, later assays would suggest that the concentration of BP-3 may have started at 20ng/ml rather than 40ng/ml.

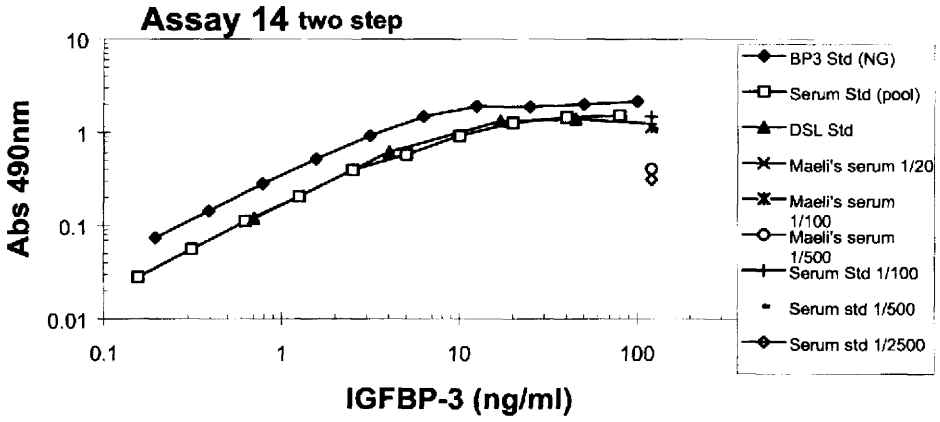
Assay 14

Aim: To extend the sensitivity of the two step assay and to assess the signal from the non glycosylated and other serum material.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	 100µl @ 100ng/ml → 0.195ng/ml (2 fold dlns) 100µl @ 80ng/ml → 0.156ng/ml (2 fold dilns) 100µl @ 1/100, 1/500, 1/2500 dilns 100µl @ 0.7, 4, 17, 45, 120ng/ml 100µl @ 1/20, 1/100, 1/500 dilns
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/4000 diln
INCUBATION STEPS	2 step: 2hrs then 1hr

Results:



Findings: The sensitivity of this assay appears to be repeatable down to a concentration of 0.156ng/ml and is highly reproducible.

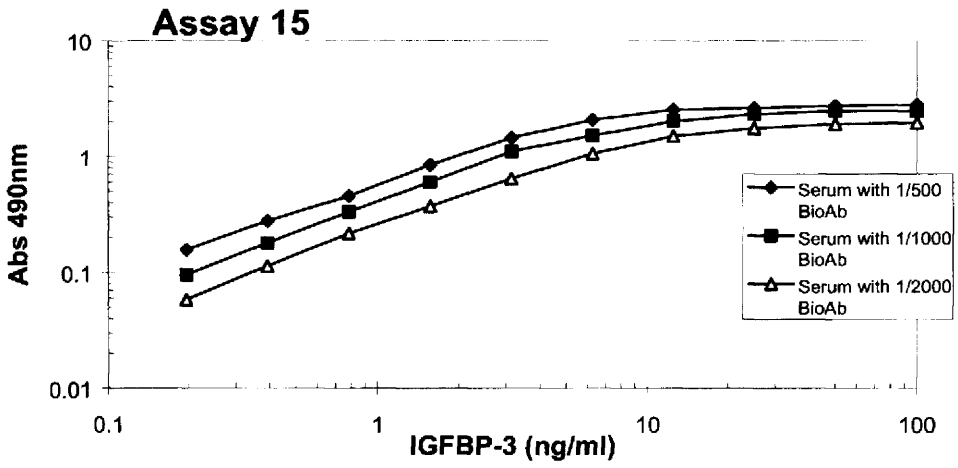
Assay 15

Aim: To determine how different concentrations of biotinylated antibody will affect the two step assay sensitivity.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0NG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL DSL STD MAELI'S SERUM	100µl @ 100ng/ml → 0.195ng/ml (2 fold dilns)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/500, 1/1000, 1/2000 dilns
INCUBATION STEPS	2 step: 2hrs then 1 hr

Results:



Findings: The absorbance readings obtained from using the 1/1000 diln of biotinylated antibody give an adequate response.

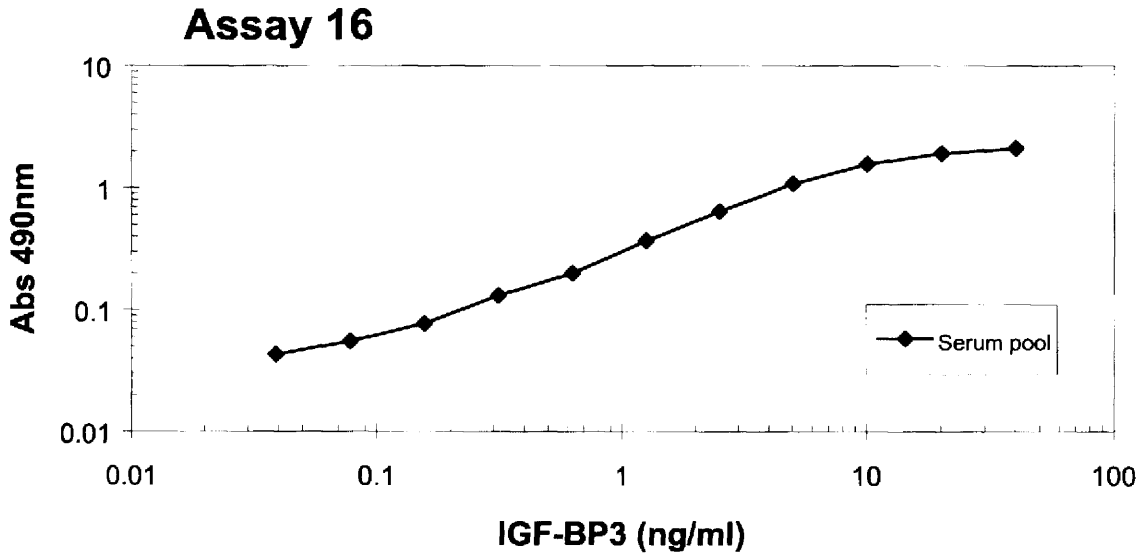
Assay 16

Aim: To assess the cross reactivity of the antibody with IGF-I and IGF-II.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL IGF-I (87/518) (3.1µg/ml) IGF-II (96/538) (5µg/ml) DSL STD MAELI'S SERUM	 100µl @ 40ng/ml → 0.0488ng/ml (2 fold dilns) 100µl @ 1.0 µg/ml → 0.0156ng/ml (2 fold dilns) 100µl @ 1.0µg/ml → 0.0156ng/ml (2 fold dilns)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/1000 diln
INCUBATION STEPS	2 step: 2hrs then 1hr

Results:



Findings: IGF-I and II did not give a significant signal at any concentration and do not therefore appear to be binding to the antibody.

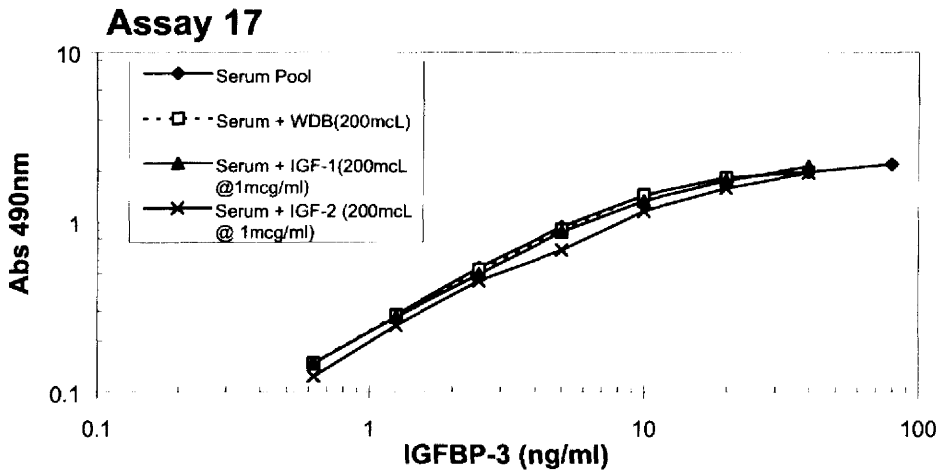
Assay 17

Aim: To assess possible competitive binding of IGF-I and II with BP-3 by spiking the serum with known amounts of the growth factors.

Method:

COATING ANTIBODY (FPLC-RBP3/3)	100µL @ 1.0 µG/ML
SAMPLES/ STANDARD NG IGFBP-3 (93/560) (3.5µg/ml) SERUM POOL SERUM WITH WDB SERUM WITH IGF-I SERUM WITH IGF-II	100µl @ 80ng/ml → 1.25ng/ml (2 fold dilns) 100µL @ 20ng/ml → 0.625ng/ml (2 fold dilns) 100µl @ 40ng/ml → 0.625ng/ml (2 fold dilns) 100µl @ 40ng/ml → 0.625 ng/ml (2 fold dilns)
BIOTINYLATED ANTIBODY (FPLC-RBP3#5)	100µl @ 1/1000 diln
INCUBATION STEPS	2 step: 2hrs then 1hr

Results:



Findings: IGF-1 and II appear to be competing with the antibody in binding to BP-3 however there does not appear to be a significant effect on absorbance levels and binding appears to be constant.

2.3.4 Discussion

Optimal conditions were found to be with the two-step incubation, whereby the samples were allowed to bind to the walls of the plate for 2 hours before the Biotinylated Antibody was added, this appeared to remedy the hook affect seen with a one step incubation of 3 hours. The standard curve established was found to be highly reproducible. However as serum must be screened before use at NIBSC facility we were unable to assess the assay on more than one individual serum sample.

In order to validate this assay for future use of the clinical assessment of patients, many serum samples would need to be tested and the assay would need to be compared with commercial ELISAs that use human serum for production of standard curves. Although initial results were encouraging this ELISA was left at the experimental stage as there would need to be considerable testing before it could be published as a standard ELISA for IGFBP3 and used for the analysis of samples in subsequent studies.

It was not therefore used for the analysis of IGFBP-3 in this thesis, and represents early stage assay development.

2.4 Cell Culture

MCF-7 and MDA MB -231 cells (kindly donated by Ludwig cancer research group) were maintained in Phenol Red IMEM (GIBCO) supplemented with 5% FCS (GIBCO), 1% L-Glutamine (sigma) and 1% Fungizone (GIBCO) in 5% CO_2 at 37°C. Conditions involved growing cells in phenol red free IMEM supplemented with 5%FCS for 72 hours prior to plating. Cells were then seeded in 96 well plates (NUNC) at 1x 10^4 cells per well in 5% DCC-FCS (Dextran charcoal coated foetal calf serum) phenol red free IMEM and incubated for 24 hours. Medium was changed to serum free IMEM for another 24 hours before the compounds were added. Stock solutions of tamoxifen, Genistein (4,5,7-trihydroxyisoflavone), Daidzein (4,7-dihydroxyisoflavone), Equol and 17β-estradiol (Sigma) in ethanol, were used to produce the required concentrations of compounds in culture medium. Control conditions also contained the appropriate amount of ethanol (0.1%).

2.5 BrdU assay

The principle of the assay was to determine cell proliferation by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis in proliferating cells. This assay indicates growth rate as opposed to cumulative cell growth. Conventional methods use thymidine, however this technique is based on the incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU). The BrdU assay was performed using a colorimetric ELISA kit (Biotrak version 2 from Amersham Pharmacia Biotech).

The assay involved culturing cells in the presence of the respective test substances in a 96-well microtitre plate at 37°C for 1 to 5 days. BrdU was added

to the cells and reincubated (usually 2-24h). During this labelling period, the pyrimidine analogue BrdU wass incorporated in place of thymidine into the DNA of proliferating cells. After removing the culture medium, the cells were fixed and the DNA denatured by addition of fixative (the denaturation of the DNA was necessary to improve the accessibility of the incorporated BrdU for detection by the antibody). The peroxidase-labelled anti-BrdU bound to the BrdU incorporated in newly synthesised, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant colour read at 450nm in a microtitre plate spectrophotomoter. The absorbance values correlated directly to the amount of DNA and thereby to the number of proliferating cells in culture (Amersham-Pharmacia-Biotech, 1998).

2.5.1 BrdU incorporation

After 24 hours of treatment with test compounds, 5-bromo-2-deoxyuridine (BrdU) was added to 200μl of medium to give a final concentration of 10μM per well. Incorporation was allowed to proceed for a further 24 hours at 37°C before the medium was collected and cells were fixed with an ethanol-containing fixative. Cells were blocked then incubated for 30 min at room temperature on a plate shaker with a peroxidase conjugated mouse monoclonal antibody (1:100). After washing, peroxidase substrate (tetramethylbenzidine) was added and the reaction stopped after 10 min with 1 M H_2SO_4. Absorbance was read within 5 min at 450nm using a 96-well plate reader. Results were from five replicate wells with experiments performed two to three times.

2.5.2 Optimal conditions

To develop conditions whereby assay blank and non specific binding control absorbance readings were low and the difference between controls and stimulated cells were optimal, the following parameters were manipulated:

1. Different cell types were used to establish which growth patterns were more suited to the assay. These included T47D and ZR75 breast cancer cell lines which were both unsuitable due to slow and for ZR75 non-monolayered growth which made denaturing and fixing cells more difficult.
2. Seeding density was altered between 1 x 10^3 cells/well and 5 X 10^5 cells/well with 1 x 10^4 cells/well giving optimal results.
3. Percentage of DCC-FCS used for seeding the cells was varied between 1%, 2.5% and 5% with optimal results seen with 5% DCC-FCS.
4. Length of time cells were starved in 0% DCC-FCS was varied between 0, 24 and 48 hours with 24 hours giving optimal results.
5. Percentage of DCC-FCS used after starvation was varied between 0%, 1%, 2.5% and 5% with compounds added to 0% starvation medium giving optimal results.
6. Test compounds were added for 3, 6 24 and 48 hours before BrdU was incorporated, with 24 hour incubations giving optimal results.
7. BrdU incubation was varied to 3, 6 and 24 hours with optimal results seen after 24 hours.
8. Cells were fixed for 30 and 60 minutes with 30 minutes giving optimal results.
9. Incubation with the blocking buffer was varied between 30 and 60 minutes with 30 minutes giving optimal results.

10. Incubation time with the Peroxidase labelled antibody was varied between 30 and 90 minutes with 30 minutes giving optimal results.
11. Incubation with the substrate TMB was varied between 5 and 30 minutes with optimal results seen after 10 minutes.

CHAPTER THREE – THE EFFECT OF TAMOXIFEN ON IGF STATUS IN BREAST CANCER PATIENTS – A CASE CONTROL STUDY

3.1 Introduction

Breast cancer is the most prevalent cancer in women in Western populations. The risk of breast cancer increases greatly with age, and other established hormone related risk factors include early menarche, late age at menopause, and delayed age at first pregnancy (Bernstein & Ross, 1993). Family history is also important, although established genetic factors only account for about 5% of breast cancers in Western populations (McPherson et al., 2000).

Insulin-like growth factor-1 is a potent mitogen for breast cancer cell lines (Karey & Sirbasku, 1988). Increased concentrations of IGF-1 have been found in patients with breast cancer when compared with healthy controls (Bruning et al., 1995). In addition, healthy subjects with high IGF-1 concentrations, when controlled for binding proteins, have been shown to have an increased risk of breast cancer (RR 7.28, highest vs. lowest quintile) (Hankinson et al., 1998), and this has been confirmed by meta-analysis (Renehan et al., 2004). It is therefore now largely accepted that high circulating levels of IGF-1 and possibly BP-3 are a risk factor for pre-menopausal breast cancer (Del Giudice et al., 1998; Johansson et al., 2004; Krajcik et al., 2002; Li et al., 2001; Renehan et al., 2006; Renehan et al., 2004; Shi et al., 2004; Toniolo et al., 2000; Yu et al., 2002).

Tamoxifen, an anti-oestrogen in use for over 20 years or more, is the most commonly used agent in the treatment of hormone responsive breast cancer

(Katzenellenbogen et al., 1997). Clinical trials have confirmed the benefit of tamoxifen in preventing breast cancer recurrence and improving disease-free survival (1988; Baum, 1998). Tamoxifen has been tested as a preventative agent in healthy women and appears to significantly reduce both invasive and non-invasive breast cancer in those at increased risk of the disease (Fisher et al., 1998; Powles et al., 1998).

Apart from its main action via oestrogen receptors, tamoxifen has been found to possess numerous other plausible mechanisms in controlling tumour growth, such as binding to protein kinase C (O'Brian et al., 1985), inhibiting ornithine decarboxylase activity (Thomas et al., 1989) and inhibiting angiogenesis (Gagliardi & Collins, 1993). Tamoxifen can also act as an antioxidant (Wei et al., 1998), and can suppress the production of TGFα and stimulate the production of TGFβ (Carmichael et al., 2000; Winston et al., 1994).

In addition, tamoxifen initially seems to reduce IGF-1 concentrations in breast cancer patients (Pollak et al., 1990) and increase concentrations of its binding proteins, but studies with longitudinal sampling and long-term follow-up have yet to be carried out.

The aim of the study was to compare concentrations of IGF-1 and its binding proteins in patients with breast cancer (n=14) with those found in age and sex matched control subjects (n=23). A further aim was to assess the longitudinal effect of tamoxifen on IGF status in patients with breast cancer.

Study published in (Campbell et al., 2001).

3.2 Materials and methods

Non-fasting serum samples were collected before surgery from patients with stage I-III invasive breast cancer, both pre-menopausal and post-menopausal. Serum samples were collected, again non-fasting, after <9, <18 and <27 months of tamoxifen treatment at routine surgical outpatients appointments. These 14 patients were part of a larger prospective study looking at the role of diet and lifestyle in survival after breast cancer. They formed a subset of women where blood samples had been collected prior to surgery, before tamoxifen treatment had begun. The baseline concentrations were compared with age- and sex-matched control subjects (n=23) taken from the general population. In the patients with breast cancer, no patient showed evidence of metastatic disease during the treatment period. None of the patients suffered from any other endocrine or malignant disorder during the treatment period. All patients had been prescribed 20mg of tamoxifen daily and gave written informed consent to participate. The study was approved by the Ethical Committee of the Royal Free and University College London Medical School.

3.2.1 Laboratory methods

Serum was stored at –80°C until analysis. Concentrations of IGF-1, IGF binding protein (IGFBP-1), and IGFBP-3 were measured in serum by means of enzyme linked immuno-sorbent assay kits (ELISA) kits purchased from Diagnostics Systems Laboratories (London, UK).

See Chapter two for further detail of Laboratory Methods.

3.2.2 Statistical methods

Owing to the small numbers of subjects involved, non-parametric tests were used throughout. The IGF status of patients with breast cancer compared with healthy controls was analysed using a Mann Whitney-U test. Analysis of the change in IGF status at particular time points compared with baseline values was carried out using Wilcoxon signed rank tests. Test results were considered to be significant when $p<0.05$.

3.3 Results

The mean (SE) patient age was 51.0 (2.7) years (range, 38-70). Five of the 14 pateints were over 50 y.

Concentrations of IGF-1, IGFBP-1 and IGFBP-3 at baseline did not differ between cases and controls **(Table 3.3.1)**. Patient concentrations at baseline and during tamoxifen treatment can be seen in **(Table 3.3.2)**.

The concentrations of the three variables measured were within the expected range reported in the literature. IGF-BP1 was increased significantly after 18 and 27 months of tamoxifen treatment **(fig 3.3.1)**. IGFBP-3 was significantly increased from baseline at nine, 18 and 27 months **(fig 3.3.2)**. IGF-1 was not decreased significantly **(fig 3.3.3)**, but the IGF-1 to IGFBP-3 ratio, a commonly used measure of bioavailable IGF-1 (Hankinson et al., 1998), was significantly decreased both at nine and 18 months **(fig 3.3.4)**. The IGF-1 to IGFBP-3 ratio was not significantly different from baseline at 27 months.

Table 3.3.1: Baseline concentrations of insulin-like growth factor 1 (IGF-1), IGF binding protein 1 (IGFBP-1), and IGFBP-3 in patients and controls

	Patients (n=14)	**Controls (n=23)**	**P value**
IGF-1 (ng/ml)	166.6(42.0)	194.2 (63.4)	NS
IGFBP-1 (ng/ml)	13.9 (2.9)	17.8 (3.9)	NS
IGFBP-3(ng/ml)	3119 (507)	3211 (598)	NS

NS, not significant. Mann Whitney-U test

Table 3.3.2: Patient concentrations of insulin-like growth factor 1 (IGF-1), IGF binding protein 1 (IGFBP-1), and IGFBP-3 at baseline and during tamoxifen treatment

	Pre-Tamoxifen baseline (n=14)	**<9 months Tamoxifen (n=11)**	**<18 months Tamoxifen (n=12)**	**<27 months Tamoxifen (n=5)**
IGF-1 (ng/ml)	166.6 (42)	144.3 (35.8)	149.4 (44.7)	181 (26.9)
IGFBP-1 (ng/ml)	21.6 (16.6)	38.9 (23.4)	52.0 (41.8)*	40.7 (24.9)*
IGFBP-3 (ng/ml)	3119 (507)	3673 (476)**	3445 (634)*	3409 (501)*

* P<0.05, **P<0.01 using Wilcoxon signed rank test compared with pre-tamoxifen baseline concentrations

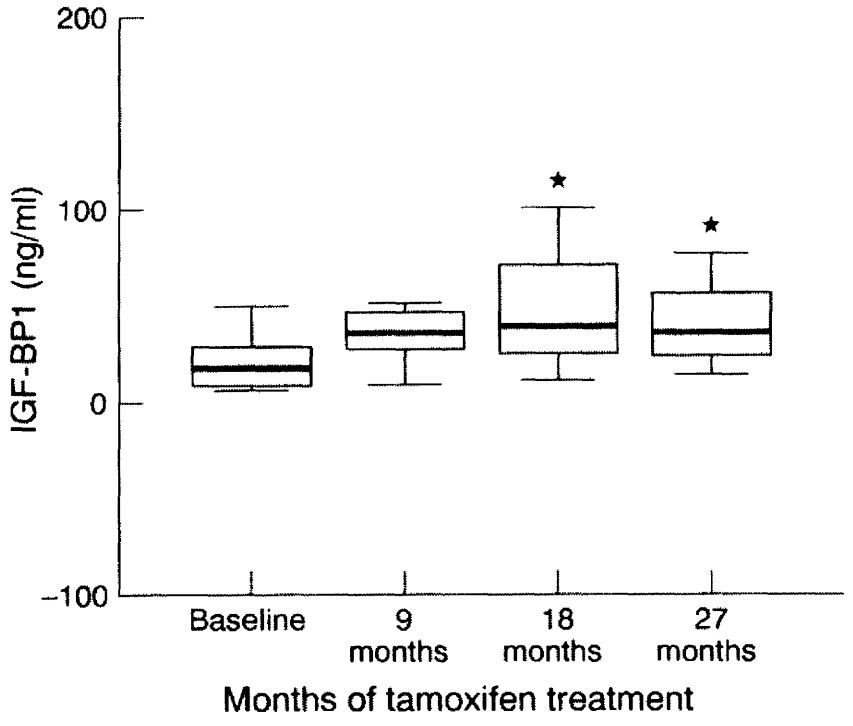


Figure 3.3.1: Effect of tamoxifen supplementation on insulin-like growth factor binding protein 1 (IGFBP-1).

Values are median (black line) with interquartile ranges (boxed area) and minimum-maximum values (bars). *p <0.05 for differences from baseline; Wilcoxon signed rank test.

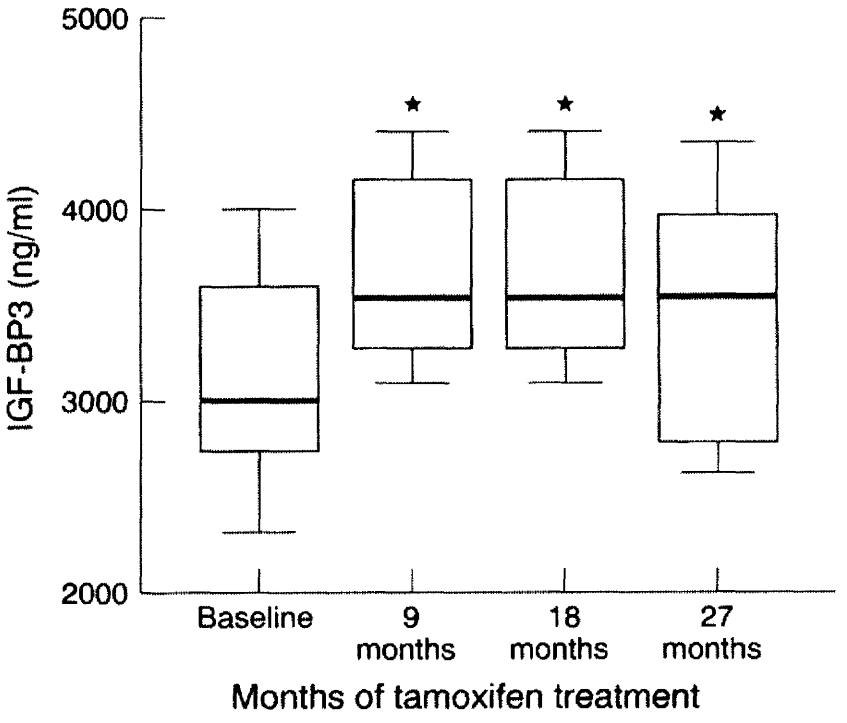


Figure 3.3.2. Effect of tamoxifen supplementation on insulin-like growth factor binding protein 3 (IGFBP-3).

Values are median (black line) with interquartile ranges (boxed area) and minimum-maximum values (bars). *$p<0.05$ for differences from baseline; Wilcoxon signed rank test.

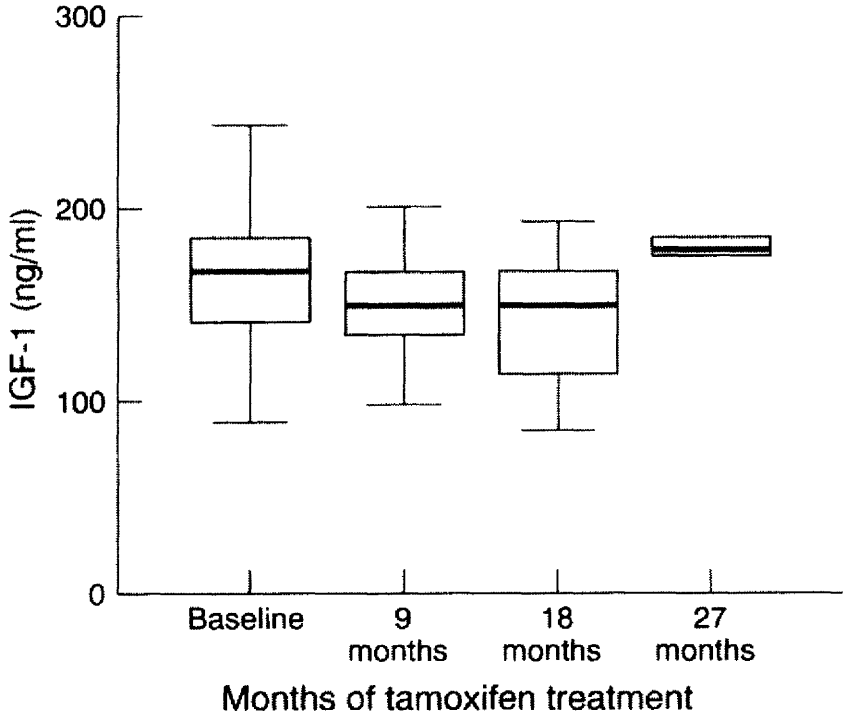


Figure 3.3.3: Effect of tamoxifen supplementation on insulin-like growth factor 1 (IGF-1).

Values are median (black line) with interquartile ranges (boxed area) and minimum-maximum values (bars). *$p<0.05$ for differences from baseline; Wilcoxon signed rank test.

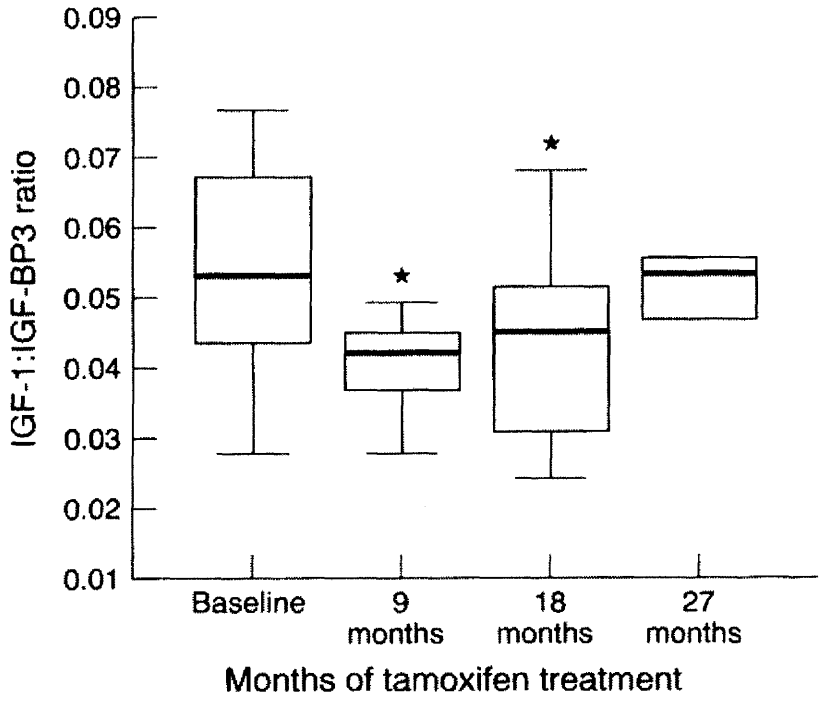


Figure 3.3.4: Effect of tamoxifen supplementation on insulin-like growth factor 1 (IGF-1) to IGF binding protein 3 (IGFBP-3) ratio.

Values are median (black line) with interquartile ranges (boxed area) and minimum-maximum values (bars). *$p<0.05$ for differences from baseline; Wilcoxon signed rank test.

3.4 Discussion

We found that tamoxifen has the ability to lower bioavailable IGF-1 (calculated as the IGF-1 to IGFBP-3 ratio) for at least 18 months. Although concentrations of IGF-1 were not reduced significantly, concentrations of its major binding protein IGFBP-3 increased significantly, thereby reducing the amount of IGF-1 available. However, this ratio of IGF-1 to IGFBP-3 was not significantly reduced from baseline at 27 months, so that the effect of longer treatment remains to be elucidated. Tamoxifen also significantly increased concentrations of IGFBP-1 from baseline after 18 months of treatment. This increase has also been seen in other studies (Helle et al., 1996; Lahti et al., 1994).

Our finding of a lack of effect on IGF-1 concentrations is surprising because other studies have shown a reduction in IGF-1 by tamoxifen (Helle et al., 1996; Pollak et al., 1990). This might be the result of the small numbers of patients in our study group or population selection. However, another study (Lahti et al., 1994) also failed to show an effect on IGF-1 at a median follow up of 29 months. These investigators had seen a significant decrease in IGF-1 values after six months of tamoxifen treatment and their data point to a limited effect after long term treatment. Our data also indicate an initial (although non-significant) reduction in IGF-1, which is lost as follow up time increases. This indicates a potentially important effect of duration of treatment on outcome and highlights the need for further longitudinal studies with strictly timed follow up periods.

We also found no differences between cases and controls in terms of IGF-1, IGFBP-1, and IGFBP-3 concentrations. Again, this is surprising because earlier studies have revealed differences in similar groups. A lack of such a finding may again simply reflect a lack of numbers. We were unable to divide our

subjects into pre-menopausal and post-menopausal groups, again owing to the small sample size, and this may have been reflected in our results because IGF-1 values have been shown to be a stronger predictor of breast cancer risk in pre-menopausal than in postmenopausal women (Hankinson et al., 1998).

Our study was not placebo-controlled, and therefore subjects were not randomised to a treatment regimen; a placebo-controlled study would have distinct design advantages over the one described here. However, placebo-controlled trials of tamoxifen would now be considered ethically unsound (Fisher et al., 2000). An earlier placebo controlled, long term study showed a significant reduction in IGF-1 after a mean follow up of 27 months (minimum follow up three months) but longitudinal samples were not taken (Pollak et al., 1990). The samples used in our study were non-fasting and this may be important because IGF values may fluctuate according to nutrient intake (Clemmons & Underwood, 1991).

Changes in serum concentrations of IGF-1 have been shown to correlate positively with serum oestradiol concentrations in menstruating women (Massa et al., 1993). This agrees with findings that IGF-1 can vary over the menstrual cycle, with significantly lower values being found during the menstrual phase than the follicular and luteal phases (Helle et al., 1998). Although other studies found that IGF-1 concentrations were dependent on the menstrual cycle phase they considered this to be a fairly minor variation (Juul et al., 1997). Our study did not take into account the effect of the menstrual cycle and it may be important to control for this in future work.

The mechanism by which tamoxifen alters IGF status has not been fully elucidated. However, tamoxifen is thought to alter IGF-1 values by reducing the production of growth hormone from the pituitary, thereby lowering the amount of

IGF-1 that is produced by the liver and released into the circulation. Tamoxifen also has direct action as an antioestrogen on breast cancer cells and appears to alter the amount of IGF-1 and binding proteins released by the cells themselves (Pollak et al., 1990; Winston et al., 1994).

Since this work was published, there have been significant developments in the field, both in the use of SERMS and other treatments for breast cancer, and in how these treatments affect IGF concentrations. These will now be summarised. Tamoxifen has been shown to reduce breast cancer incidence by 30-40% in high risk subjects, in large phase III Trials (Cuzick et al., 2002; Fisher et al., 1998; Gasco et al., 2005; Veronesi et al., 2003). However, with the increased risk of endometrial cancer and venous thrombolitic events attributed to tamoxifen other compounds including lower dose tamoxifen, other SERMS, aromatase inhibitors, and retinoids are now being assessed. Some of these trials have included analysis of IGF-1 and its binding proteins as cancer risk biomarkers.

Low dose tamoxifen studies have shown that 10mg/day gives comparable reductions in IGF-1 levels to standard 20mg/day doses (Bonanni et al., 2001; Decensi et al., 1998; Decensi et al., 1999; Decensi et al., 2003) with possible dose-resposnse relationships seein in IGF-1/BP-3 rations (Bonanni et al., 2001). A recent study by (Guerrieri-Gonzaga et al., 2006) has shown a 13% reduction in IGF-1 levels after 40 months of 5mg/day tamoxifen in 235 pre-menopausal women at risk of breast cancer, while the use of low does tamoxifen for combination hormone replacement therapy users has shown a significant reduction in IGF-1 and increase in BP-3 for those who received 5mg/day tamoxifen over 12 months (Decensi et al., 2007).

Other SERMS including Raloxifene have been trialed in large studies and show 76% reduced risk of breast cancer with no increase risk in endometrial cancer (Cauley et al., 2001; Martino et al., 2004) with the National Surgical Adjuvant Breast and Bowel Project Study of tamoxifen and Raloxifene trial (STAR) showing that Raloxifene is as effective as tamoxifen in reducing the risk of invasive breast cancer and has a lower risk of thromboembolic events and cataracts but a nonstatistically significant higher risk of non-invasive breast cancer(Vogel et al., 2006). Raloxifene has also been shown to reduce IGF-1 and increase BP-3 when compared with placebo after 3 months (Andersson et al., 2002; Eng-Wong et al., 2003) and 2 years in post-menopausal type II diabetes patients (Duschek et al., 2004).

Recent trials have also shown improved efficacy and decreased side effects of aromatase inhibitors such as anastrazole and letrazole compared to tamoxifen (Baum et al., 2002; Ellis et al., 2001; Howell et al., 2005; Mouridsen et al., 2001; Nabholtz et al., 2000). However, the effects of Vorozole on IGF-1 levels (Harper-Wynne et al., 2002) showed a non significant increase in IGF-1 and are in line with earlier studies showing increases in IGF-1 levels with other aromatase inhibitors (Bajetta et al., 1997) (Frost et al., 1996).

A case control study found that one year of the retinoid fenretinide reduced IGF-1 by 13% in women aged <50 and by 3% in women over 50 with changes maintained for up to 5 years (Decensi et al., 2001). However, other trials comparing the retinoid fenretinide with tamoxifen found only 2% lowering in IGF-1 with Fenretinide vs 15% in tamoxifen and no synergistic effects between the two treatments were seen (Guerrieri-Gonzaga et al., 2006).

3.5 Conclusion

Patients with breast cancer had similar concentrations of IGF-1, IGFBP-1, and IGFBP-3 to those seen in healthy control subjects in this pilot study. Tamoxifen can increase IGFBP-1, IGFBP-3, and reduce the IGF-1 to IGFBP-3 ratio, and these alterations may contribute to the therapeutic benefits of tamoxifen. The long term effects of tamoxifen use on IGF status remain to be established. Longer follow up with larger numbers of patients would determine when, and for how long, tamoxifen can reduce circulating IGF-1. The effect of other treatments for breast cancer, including lower dose tamoxifen and other SERMS is now being tested. Given the increase in risk of breast cancer associated with higher levels of IGF-1 in pre-menopausal women and the ability of phytoestrogens to act as SERMS, the potential of these dietary compounds to alter IGF status like tamoxifen and other SERMs is investigated in the following chapters.

CHAPTER FOUR – SHORT-TERM PHYTOESTROGEN SUPPLEMENTATION IN HEALTHY FEMALE VOLUNTEERS – PILOT STUDY

4.1 Introduction

Phytoestrogens have been proposed to protect against many diseases including hormone-dependent cancers and coronary heart disease. Found in many plant products, but particularly soy, legumes, and vegetables, they have a wide variety of proposed biochemical effects including estrogenic and antioxidant effects (Bingham et al., 1998).

Many studies have examined the effect of phytoestrogen supplementation *in vitro*, but fewer have looked at their physiological effects *in vivo.* A summary of human intervention studies examining effects of phytoestogen supplementation on IGF-1 levels can be found in table1.3.5.1. As a preliminary pilot *in* vivo study, we have examined the effects of a single intake of phytoestrogens and a week-long intervention. We have assessed a variety of biochemical endpoints, including phytoestrogen metabolites in urine, antioxidant status and lipid status. These are all potentially altered by phytoestrogen consumption (Bingham et al., 1998). We have also examined the effects of phytoestrogen supplementation on insulin-like growth factor (IGF) profile as there is increasing evidence that high circulating levels of IGF-1 and possibly BP3 are a risk factor for pre-menopausal breast cancer (Del Giudice et al., 1998; Hankinson et al., 1998; Johansson et al., 2004; Krajcik et al., 2002; Li et al., 2001; Renehan et al., 2006; Renehan et al., 2004; Shi et al., 2004; Toniolo et al., 2000; Yu et al., 2002). As shown in the previous chapter, levels of IGF-1 and its related binding proteins are altered by

tamoxifen (Campbell et al., 2001), and phytoestrogens have a similar structure to tamoxifen (Bingham et al., 1998).

We have also examined the effects of phytoestrogens on DNA damage, as assessed by the COMET assay. Phytoestrogens have antioxidant properties (Bingham et al., 1998) and may therefore protect against oxidative DNA damage.

The aim of this pilot feeding study was therefore to compare the effects of acute (single 80 mg load) versus chronic (80 mg/day for 7 days) administration of phytoestrogens on a number of physiological endpoints including urinary phytoestrogen metabolites, lipids, antioxidant status, DNA damage and IGF-1, IGFBP-1 and IGFBP-3, in healthy female volunteers (n=10).

This was a feasibility study used to establish the assays for analyses of serum IGF-1, BP1 and BP3 concentrations and GC-MS analysis of phytoestrogens in urine and to determine the practicalities of use of the phytoestrogen-rich 'energiser bar' for future feeding studies.

Study published in (Woodside et al., 2006)

4.2 Materials and methods

4.2.1 Study Design

Subjects were healthy female volunteers (n=10), employed within University College London. The study was fully explained to the subjects, and written informed consent obtained. This study received ethical approval from the Royal Free and University College London Medical School Research Ethics Committee.

The supplement used was a soy and linseed bar, each bar containing a total of 20mg of isoflavones and lignans. The nutritional information is shown in **Table 4.2.1.1.**

The ingredients list was as follows: Dehulled Split Soya Beans, Glucose Syrup, Sugar, Golden Linseeds, Rye Flour, Crisped Rice, Dried Apple, Rolled Oats, Fructose, Corn Syrup Solids, Water, Flavourings and Pectin.

Table 4.2.1.1: Estimated nutritional content of phytoestrogen supplement

	per 100g	per bar	Per day
Energy (kJ)	1656	497	1988
Energy (Kcal)	395	119	476
Protein (g)	15.1	4.5	18
Carbohydrate (g)	56.7	17.0	68.0
of which sugars (g)	29.2	8.8	35.2
Fat (g)	12.0	3.6	14.4
of which saturates (g)	1.9	0.6	2.4
Fibre (g)	4.2	1.3	5.2
Sodium (g)	0.1	Trace	0.03

The study was divided into two phases with a wash-out period of 6 days between phases. Subjects were instructed to follow a soy-free diet for 1 week before the study began.

4.2.2 Phase 1

Subjects were asked to provide a 24-h urine collection prior to the study. After an overnight fast, subjects gave a baseline blood sample. They were then given a breakfast containing 80 mg of phytoestrogens (4x supplement bars). Blood samples were taken at 8 and 24 h (fasting) after the meal with spot urine samples collected during the hours 1-2, 2-4, 4-6, 6-8 and 24 h. Meals or snacks, excluding soy products, were permitted throughout the 8 h post meal period, but only liquids were consumed until 2 h after the meal.

4.2.3 Phase 2

Six days later, subjects collected a further 24-h urine sample and had a further fasting baseline blood sample prior to the study. They were then asked to take 80 mg Phytoestrogen (4x supplement bars) daily for a period of 7 days, after which they collected a final 24-h urine sample and had a final fasting blood sample taken.

4.2.4 Collection and preparation of biological samples

Urine samples (24 h) were stabilised with 1-2 g/l ascorbic acid and sodium azide (0.1 g/L) and stored at -80°C until analysis. Spot urine samples were not stabilised but immediately frozen after collection. Blood samples either kept at 4°C for a maximum of 30 min (plasma) or were allowed to clot for 30 minutes in the dark (serum) and then centrifuged at 3000 rpm for 10 min. Serum or plasma was aliquoted and stored at -80°C until analysis. Lymphocytes were collected into a Vacutainer Cell Preparation Tube (Becton Dickinson, New Jersey), extracted and washed according to manufacturer's instructions and stored at -80°C.

4.2.5 Endpoint measurements

Urine samples were analysed for the isoflavonoids genistein and daidzein, the isoflavan equol and the lignans enterolactone and enterodiol levels, using gas chromatography-mass spectrometry following enzyme hydrolysis, extraction into ether and silylation according to Morton et al. (Morton et al., 1994). Ascorbic acid concentrations were determined in EDTA plasma as described by Vuillemier & Keck (Vuillemier & Keck, 1989). Concentrations of lipid-soluble antioxidants were measured by HPLC in serum according to the method of

Thurnham et al. (Thurnham et al., 1988). Levels of aqueous phase lipid hydroperoxides were assessed in plasma by the FOX 1 (ferrous oxidation in xylenol orange) assay according to Wolff (Wolff, 1994). Susceptibility of LDL to oxidation was assessed in Phase 2 according to McDowell et al. (McDowell et al., 1995). DNA damage was analysed by the COMET assay in both lymphocytes according to Spanswick et al. (Spanswick et al., 1999).

Serum total cholesterol was estimated using an enzymatic CHOD-PAP kit, while serum triglycerides were measured using the Peridochrom GPO-PAP kit (both Boehringer Mannheim). Precipitation for HDL-cholesterol estimation employs phosphotungstic Mg^{2+} reagents.

Serum samples were analysed for IGF-1 and IGFBP-1 and IGFBP-3 using ELISA kits from Diagnostic System Laboratories (London, UK). For all laboratory methods, daily quality controls were utilised to ensure both within- and between-assay reproducibility.

See Chapter two for further detail on Methods.

4.2.6 Statistical methods

Comparisons between subjects pre- and post-intervention were carried out using a non-parametric Wilcoxon signed rank test due to the small numbers of subjects and the non-normal distribution of the data. Associations between continuous variables were tested using Spearman correlation coefficients. All analyses were carried out using SPSS.

4.3 Results

Subjects had a mean age of 33.1 years ranging from 23 to 50. Eight were pre-menopausal, of whom three were on the oral contraceptive pill. One of the

subjects was post-menopausal and was also on hormone replacement therapy. All subjects were non-smokers.

Concentrations of genistein, daidzein, equol, enterolactone and enterodiol were elevated, both after a single 80 mg dose **(Table 4.3.1)** and after 1 week of supplementation **(Table 4.3.2).**

The effect of phytoestrogen supplementation on lipid and antioxidant-related endpoints is shown in **Table 4.3.3**. Neither acute nor chronic phytoestrogen supplementation had any effect on any of the lipid or antioxidant-related endpoints assessed.

All IGF-1 and IGFBP-3 levels fell within the range of expected values given for healthy females in this age group. As fasting samples are required for these assays, measures of IGF-1 and IGFBP-3 status were limited to baseline and 24-hour post supplement in the first phase of the study.

Levels of IGF-1 were unchanged following the single 80 mg load. However, in Phase 2, IGF-1 and IGFBP-3 concentrations were significantly elevated after a week of supplementation. Levels of IGFBP-1 were not altered by phytoestrogens in either phase of the study **(Table 4.3.4)**.

The change in IGF-1 in Phase 2 was significantly associated with the change in IGFBP-3 ($r=0.721$, $P<0.05$; Spearman correlation coefficient) **(Figure 4.3.1)**. However, if the ratio of IGF-1/IGFBP-3 was compared pre- and post-supplementation, the ratio was also significantly increased post-supplementation, suggesting that IGF-1 has increased independently of the increase seen in IGFBP-3 **(Table 4.3.4)**.

Table 4.3.1: Effect of one phytoestrogen meal on urinary phytoestrogen excretion- Phase 1

Baseline [median (IQ range)]		1–2 h [median (IQ range)]	2– 4 h [median (IQ range)]	4– 6 h [median (IQ range)]	6– 8 h [median (IQ range)]	24 h [median (IQ range)]
Genistein (ng/ml)	196 (146, 308)	217 (169, 377)	313 (201, 416)*	392 (344, 558)*	1257 (1013, 2711)*	289 (139, 417)
Daidzein (ng/ml)	428 (236, 593)	484 (388, 714)*	642 (375, 854)*	952 (681, 1837)*	3283 (2463, 4365)*	408 (252, 634)
Equol (ng/ml)	91 (48, 192)	104 (31, 268)	191 (25, 328)	356 (36, 523)*	757 (207, 915)*	136 (42, 264)
Enterolactone (ng/ml)	284 (105, 334)	280 (149, 307)	226 (153, 308)	242 (146, 305)	301 (206, 335)	284 (169, 461)
Enterodiol (ng/ml)	69 (22, 101)	66 (42, 88)	79 (47, 88)	77 (40, 95)	75 (48, 95)	77 (46, 145)

* P<.05 when compared to baseline, Wilcoxon signed rank test.

Table 4.3.2: Effect of 1 week of phytoestrogen supplementation on urinary phytoestrogen excretion – Phase 2

Baseline [median (IQ range)]		Postweek [median (IQ range)]
Genistein (ng/ml)	206 (88, 306)	2775 (1818, 3101)*
Daidzein (ng/ml)	385 (195, 599)	4571 (3934, 5803)*
Equol (ng/ml)	96 (53, 131)	899 (501, 1540)*
Enterolactone(ng/ml)	224 (95, 403)	392 (181, 565)*
Enterodiol (ng/ml)	46 (19, 81)	83 (58, 114)*

*P<.05 when compared to baseline 2, Wilcoxon signed rank test.

Table 4.3.3: Effect of phytoestrogen supplementation on lipids and antioxidant status

	Phase 1 [median (IQ range)]			Phase 2 [median (IQ range)]	
	Baseline	8 h	24 h	Baseline	postweek
Total cholesterol (mmol/L)	4.59 (4.07, 5.31)	4.47 (4.05, 4.82)	4.19 (3.89, 4.79)	4.63 (4.16, 5.54)	5.00 (4.32, 5.27)
HDL cholesterol (mmol/L)	1.49 (0.91, 1.68)	1.43 (0.96, 1.62)	1.43 (0.93, 1.87)	1.48 (1.16, 1.58)	1.57 (1.10, 1.73)
Triacylglycerol (mmol/L)	1.00 (0.83, 1.58)	1.44 (1.31, 2.39)	0.92 (0.66, 1.20)	1.19 (0.84, 1.78)	1.19 (0.86, 1.45)
LDL cholesterol (mmol/L)	2.59 (2.24, 3.39)	2.27 (2.13, 2.86)	2.36 (2.16, 2.51)	2.86 (2.29, 3.41)	2.96 (2.70, 3.31)
FOX1 (µmol/L)	1.65 (1.15, 2.57)	1.42 (1.37, 1.64)	1.20 (1.10, 1.37)	1.35 (1.19, 1.45)	1.34 (1.29, 1.46)
Retinol (µmol/L)	1.00 (0.74, 1.49)	0.79 (0.48, 1.47)	1.01 (0.69, 1.30)	0.99 (0.61, 1.10)	0.91 (0.52, 1.19)
α-Tocopherol (µmol/L)	20.9 (17.6, 23.9)	19.1 (14.8, 22.8)	21.4 (16.3, 24.8)	18.9 (15.5, 24.2)	21.0 (12.6, 23.3)
Lycopene (µmol/L)	0.09 (0.04, 0.16)	0.09 (0.06, 0.11)	0.09 (0.06, 0.11)	0.06 (0.04, 0.09)	0.07 (0.03, 0.16)
α-Carotene (µmol/L)	0.14 (0.06, 0.29)	0.12 (0.06, 0.21)	0.15 (0.09, 0.27)	0.08 (0.04, 0.11)	0.07 (0.05, 0.41)
β-Carotene (µmol/L)	0.14 (0.09, 0.20)	0.13 (0.09, 0.18)	0.16 (0.08, 0.19)	0.12 (0.09, 0.16)	0.10 (0.07, 0.17)
DNA damage (olive tail moment)	–	–	–	4.21 (1.50, 5.07)	2.11 (1.31, 2.74)
Lag time (min)	–	–	–	44.2 (37.4, 53.6)	44.5 (38.8, 48.4)

Table 4.3.4: Effect of phytoestrogen supplementation on insulin-like growth factor and binding protein concentrations

Phase 1 [median (IQ range)]			Phase 2 [median (IQ range)]	
	Baseline	24 h	Baseline 2	Postweek
IGF-1 (ng/ml)	218 (151, 293)	250 (179, 310)	155 (123, 258)	265 (228, 360)*
IGFBP-1 (ng/ml)	22.6 (19.0, 36.8)	17.4 (15.8, 34.6)	24.8 (14.6, 57.7)	14.7 (10.0, 36.0)
IGFBP-3 (ng/ml)	4564 (3954, 5306)	4110 (3887, 4533)	3735 (3631, 4196)	4420 (4192, 4935)*
IGF-1/IGFBP-3 ratio	0.05 (0.04, 0.06)	0.06 (0.04, 0.07)	0.04 (0.03, 0.06)	0.06 (0.05, 0.07)*

*P<.05 when compared to baseline, Wilcoxon rank signed test.

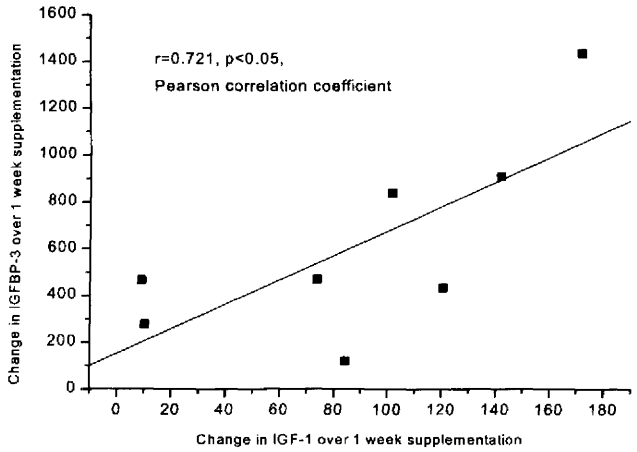


Figure 4.3.1: Association between change in IGF-1 and IGFBP-3 over 1 week of supplementation (r=0.721, P<0.05; Spearman correlation coefficient).

4.4 Discussion

This study has assessed the effect of phytoestrogen supplementation, in the form of a soy, rye and linseed bar, on a variety of biochemical endpoints. Concentrations of phytoestrogen metabolites in urine reached their highest levels 6-8 h after supplementation for isoflavones, and 24 h for lignans in phase 1. This is generally in agreement with other studies examining the effect of supplementation on urinary excretion of phytoestrogens (King & Bursill, 1998); (Rowland et al., 2003; Setchell et al., 2003). This study, however, was not designed to be a rigorous study of phytoestrogen pharmacokinetics.

Phytoestrogens can act as antioxidants (Bingham et al., 1998) and may inhibit the oxidation of LDL (Lissin & Cooke, 2000) although the data from this study has not confirmed this in terms of lag time to oxidation. A protective/antioxidant effect has been shown by phytoestrogen supplementation on the susceptibility of LDL to oxidation in another study (Tikkanen et al., 1998). Another study, however, in common with this one, has failed to show an inhibition of lipid peroxidation after 8 weeks of isoflavonoid supplementation in subjects with high-normal blood pressure (Hodgson et al., 1999), measuring urinary F-2-isoprostanes a reliable indicator of oxidative stress. The study measuring F-2-isoprostanes was randomised, double-blind and placebo-controlled, in contrast to the present study and was carried out in relatively large numbers (n=59). Our subjects were healthy and therefore had normal antioxidant status. Therefore the lag times of these subjects may not have been easily affected by this supplement.

We have also observed an apparent reduction in DNA damage in lymphocytes (Olive tail moment from 4.21 to 2.11) after 1 week of phytoestrogen supplementation, although this was not significant. We have previously shown

that genistein and equol within physiological range can reduce oxidative DNA damage *in vitro* in lymphocytes (Sierens et al., 2001). Mitchell & Collins (Mitchell & Collins, 1999) carried out a 4 week feeding study in healthy men to assess the effects of a phytoestrogen supplement in the form of soy milk on levels of DNA damage. There was no significant effect on H_2O_2-induced DNA damage in lymphocytes in those receiving the soy milk supplement as assessed by the comet assay. However, the levels of oxidised pyrimidine base damage over the 4-week period progressively decreased. A recent study found no effect of rye crispbread (rich in lignans secoisolariciresinol and matairesinol) over 2 weeks on oxidative DNA damage (Pool-Zobel et al., 2000). We have only evaluated endogenous DNA damage in this study, where the damage may or may not have been oxidative in origin, and our intervention period was shorter than that which previously showed an effect. The antioxidant potential of these foods and compounds *in vivo* clearly requires further investigation.

This study found no effect of either a single dose or week's supplementation with 80 mg phytoestrogens daily on serum lipids. This is in contrast to a meta-analysis of studies published in 1995 (Anderson et al., 1995). Of the 38 studies reviewed, 89% reported a net decrease in plasma cholesterol that averaged around 9% for an intake of around 47 g soy protein daily. However, it is notable that 77% of the studies included in the meta-analysis had 95% confidence intervals that included zero.

Since the meta-analysis, several well-controlled studies have been carried out, and the results of these have been variable (Lichtenstein, 2001). There are several potential explanations for this variability. Merz-Demlow et al. (Merz-Demlow et al., 2000) suggest that the effect of isoflavones on lipids may be related to menstrual cycle phase. The food matrix may also be important, as

Lichtenstein (Lichtenstein, 2001) suggests that the effect of isoflavones appears to be somewhat dependent on whether the isoflavones are ingested in isolation or as an enriched preparation of those endogenously present in soy.

A recent study observed a reduction in LDL cholesterol in 156 men and women by an average of 6%. This was due entirely, however, to a mean reduction of 9% among those with a raised baseline LDL cholesterol (>4.29 mmol/l) and was only observed in men and post-menopausal women (Crouse et al., 1999). The original meta-analysis also found a greater lowering effect in those with initially raised total cholesterol levels (Anderson et al., 1995). It may be that we observed no difference in our largely pre-menopausal sample set because they had low-normal lipid status initially, but it is more likely that one week of supplementation was too short to demonstrate an effect.

This study has shown that 1 week of phytoestrogen supplementation can alter IGF status, producing a significant increase in both IGF-1 and IGFBP-3 levels. The vast majority of IGF-1 is carried through the circulation bound to a complex containing IGFBP-3 and, until released by proteolysis, is relatively unreactive (Rajaram et al., 1997). Thus the strong correlation between increasing levels of IGF-1 and IGFBP-3 was as expected. However, when the ratio of IGF-1 to BP-3 was considered, this ratio was significantly increased post-supplement, and this finding requires further elucidation. Tamoxifen, a selective estrogen receptor modulator (SERM) has been shown to lower IGF-1 and increase IGFBP-3 concentrations (Campbell et al., 2001; Helle et al., 1996); therefore the phytoestrogen supplement appears to be behaving differently to tamoxifen. Oral estrogen treatment for menopausal women has also been shown to reduce IGF-1 (Kelly et al., 1993) through inhibition of hepatic IGF-1 synthesis. The mechanism by which this supplement has increased IGF-1 requires further

study. Since conducting this study in 1998, other intervention studies have also published their findings on the effects of soy supplementation on IGF status **(Table 1.3.5.1)**.

Of the twelve studies published to date, half have seen a significant increase in serum IGF-1 concentrations while the others have seen no effect **(Table 1.3.5.1).**

Two studies were conducted using soy protein with a milk protein control. In men, serum IGF-1 was higher in those supplemented with soy protein than those consuming milk protein (Khalil et al., 2002). The same supplements given to post-menopausal women (Arjmandi et al., 2003) resulted in significant increases in IGF-1 on both soy protein ($p<0.0001$) and the milk protein control ($p=0.0045$). However like the men, the greatest increases in IGF-1 (97%) was seen in women on the soy protein especially those who were not on HRT. These two studies would suggest that protein itself does increase IGF-1 but that soy protein had an effect on IGF-1 independent of its protein content. Calcium content did not explain the findings as both supplements had similar calcium content.

By contrast, in a three month study looking at markers of bone turnover (Wangen et al., 2000) in pre-menopausal women, IGF-1 and IGF-BP3 were increased by a low isoflavone diet, while in post-menopausal women, there were trends towards decreased IGF-1 and IGF-BP3 concentrations with increasing isoflavone concentration. The authors concluded that, although soy isoflavones do affect markers of bone turnover, the changes observed were of small magnitude and were not likely to be clinically relevant. In a study looking at soy protein supplementation with or with out 83 mg isoflavones (Adams et al., 2003), it was found that changes in serum IGF-1 and BP3 were similar in both

groups and there appeared to be no effect of soy isoflavones on IGF-1 when compared to the protein supplement alone. Similarly, a one year soy protein supplementation in post-menopausal women (n=62) (Arjmandi et al., 2005), found that both soy and dietary protein supplements significantly increased serum IGF-1, however only those consuming soy protein had a significant increase in IGF-BP3.

In a 2 year soya intervention (~30mg isoflavones) in pre-menopausal women (Maskarinec et al., 2005b), there was no significant change in serum IGF-1 or BP3 throughout. They did see a positive correlation between total isoflavone excretion and IGF-1 ($p=0.004$). The author concluded that adding soya foods to the diet of pre-menopausal women does not appear to lower serum levels of IGF-1 and BP-3, if anything the greater protein intake from soy may lead to a small increase in IGF-1 serum levels.

In all of the studies mentioned above it is difficult to disentangle the effects of isoflavones from soy protein (Voskuil et al., 2005) especially in those studies which have looked at soy supplementation as part of an overall healthy dietary intervention (Gann et al., 2005; Kaaks et al., 2003; Spentzos et al., 2003). Only our study (discussed in chapter four) (Campbell et al., 2004) and, to our knowledge, one subsequent study published recently (Vrieling et al., 2007), has looked at the effects of isoflavone supplementation on its own.

The results from this study and the intervention studies above are inconclusive but possibly show opposing effects of isoflavones and soy protein, with IGF-1 decreased by isoflavones but increased by soy protein and possibly protein in general. Certainly feeding studies (Clemmons et al., 1985; Smith et al., 1995) have shown that a diet restricted of certain essential amino acids reduces IGF-1 levels possibly by decreasing production of GH which in turn reduces hepatic

production of IGF-1 (Ketelslegers et al., 1995) and increases BP-1 (Jousse et al., 1998). It has been observed previously that IGF is closely linked to nutritional status (Clemmons & Underwood, 1991). Our observation of an increase in both IGF-1 and IGF-BP3 could well be attributed to the increase in kilocalories and protein consumed in the phytoestrogen bar (476 Kcal daily; 18 g protein daily; Table 1), as IGF-1 appears to be closely related to protein and energy metabolism (Clemmons & Underwood, 1991). No dietary intake data were collected in the present study so it is unclear whether total protein and energy intake increased or whether subjects unconsciously decreased their intake to compensate for the extra calories over the week. Intervention studies using a phytoestrogen extract or, as a control, a food supplement from which phytoestrogens have been removed, should be used in future to exclude the possibility that an increase in protein and/or energy intake produced the observed alteration in IGF-1 and IGF-BP3.

The status of endogenous oestrogen concentrations may also contribute to the differences seen with IGF-1 – this was a heterogeneous study population including pre and post-menopausal women, HRT vs none, those on the oral contraceptive pill, etc. Thus it may also be important to take into account the effects of the menstrual cycle in future studies as it has been shown that IGF-1 can vary throughout the menstrual cycle (Caufriez et al., 1986; Helle et al., 1998; Juul et al., 1997; Lavigne et al., 2004).

Oestrogenic, and therefore confounding, effects of oral contraceptives and hormone replacement therapy should also be considered as numbers were not large enough in this study to examine their effect.

The supplement bar we used contained two classes of phytoestrogen – isoflavones (found predominantly in soy) and lignans (found predominantly in

linseed)(Bingham et al., 1998). The biological effects of these two classes may be different, and research has, to date, concentrated on the isoflavones. However, some recent studies have assessed lignan intake and would appear to show a protective effect on breast cancer risk (Keinan-Boker et al., 2004; Thanos et al., 2006; Touillaud et al., 2007). From this study, we cannot distinguish the effect of the two different classes, or indeed, conclusively attribute the biological effects we have observed to the phytoestrogens independent of any other supplement constituent.

Nevertheless these findings may have future implications for health and disease. Phytoestrogens have been proposed to protect against breast cancer, as Japanese women, who consume their traditional diet with a high intake of phytoestrogens, have a very low incidence of breast cancer (Woodside & Campbell, 2001). IGF-1 has been shown to be a risk factor for breast cancer in pre-menopausal women (Del Giudice et al., 1998; Hankinson et al., 1998; Johansson et al., 2004; Krajcik et al., 2002; Li et al., 2001; Renehan et al., 2006; Renehan et al., 2004; Shi et al., 2004; Toniolo et al., 2000; Yu et al., 2002) and, if it can be confirmed that phytoestrogens increase circulating IGF-1 concentrations, then this would actually indicate an increased risk of breast cancer in those consuming high-phytoestrogen diets. Obviously further work would be required before such conclusions can be drawn, but the data presented here do highlight the need for further, well-designed trials. Some of the issues raised above have been taken into consideration in the study design described and tested in chapter 5.

In conclusion, this small study suggests that one week of supplementation with a phytoestrogen-rich bar alters concentrations of IGF-1 and IGF-BP3. The supplement also seemed to protect DNA against damage in lymphocytes, but

had no effect on other antioxidant parameters or lipid status in healthy female subjects. Further larger and long-term intervention studies using isolated isoflavone supplementation, which therefore allow the inclusion of a placebo control, should determine whether the isoflavone content of soy has an effect on IGF status.

CHAPTER FIVE – THE EFFECT OF ISOFLAVONE SUPPLEMENTATION ON INSULIN-LIKE GROWTH FACTOR, LIPID AND ANTIOXIDANT STATUS IN HEALTHY FEMALE VOLUNTEERS

5.1 Introduction

High concentrations of IGF-1 in serum have been associated with an increased risk of breast cancer, in case control studies (Bohlke et al., 1998; Bruning et al., 1995; Del Giudice et al., 1998; Johansson et al., 2004; Li et al., 2001; Peyrat et al., 1993; Yu et al., 2002) and more recently in prospective studies (Hankinson et al., 1998; Krajcik et al., 2002; Muti et al., 2002; Rinaldi et al., 2006; Toniolo et al., 2000). Most reviews agree that this relationship is more prominent in pre-menopausal women (Fletcher et al., 2005; Renehan et al., 2004; Schernhammer et al., 2005; Shi et al., 2004; Sugumar et al., 2004) but not all studies comply (Kaaks et al., 2002; Rinaldi et al., 2006; Rinaldi et al., 2005b), although a recent prospective analysis of the Nurses Health study II cohort study found no associations in either pre or post-menopausal women (Schernhammer et al., 2006).

Tamoxifen has been shown to lower circulating IGF-1 levels by approximately 30% in breast cancer patients (Campbell et al., 2001; Pollak et al., 1990), (Colletti et al., 1989; Helle et al., 1996; Lonning et al., 1992) and in healthy women (Bonanni et al., 2001; Decensi et al., 1998; Decensi et al., 2003). Isoflavones are a class of phytoestrogens which may have a variety of potential health benefits in endocrine-responsive cancers (Adlercreutz, 2002b), (Adlercreutz, 2002a) and CVD (Cassidy & Hooper, 2006), (Bingham et al.,

1998); (This et al., 2001). They are obtained in the diet predominantly from soy products (Bingham et al., 1998). Isoflavones are structurally similar to tamoxifen and, like tamoxifen, can act as selective oestrogen receptor modulators (Brzezinski & Debi, 1999). They may therefore be able to alter concentrations of IGF-1 and its binding proteins.

The aim of this study was to determine whether one month of isoflavone supplementation could alter serum concentrations of IGF-1 and its related binding proteins. In light of the results in the previous chapter, where a phytoestrogen-rich bar providing 80 mg phytoestrogens/day over 7 days produced an increase in IGF-1 and IGFBP-3, this study used a phytoestrogen extract, which allowed the use of a placebo control. The effect of the menstrual cycle on insulin-like growth factor profile was accounted for in the study design. We also assessed whether the isoflavone supplement produced an alteration in hormone, lipid or antioxidant status as effects on these parameters have been demonstrated in some studies (Merz-Demlow et al., 2000; Takatsuka et al., 2000), but not in others (Howes et al., 2000; Jenkins et al., 2000; Nestel et al., 1999; Simons et al., 2000). The inclusion of a food frequency questionnaire and a 7-day food diary completed in each phase allowed the assessment of nutritional intake.

Study published in (Campbell et al., 2004).

5.2 Materials and methods

5.2.1 Study design

The study was based on a randomised, double-blind, placebo-controlled cross-over design (Figure 4.2.1). Pre- and post-menopausal subjects were randomised to receive either two placebo or two supplement tablets containing

a total of 80 mg isoflavones daily. Each phase was followed by a minimum 2-month washout period and the alternative intervention.

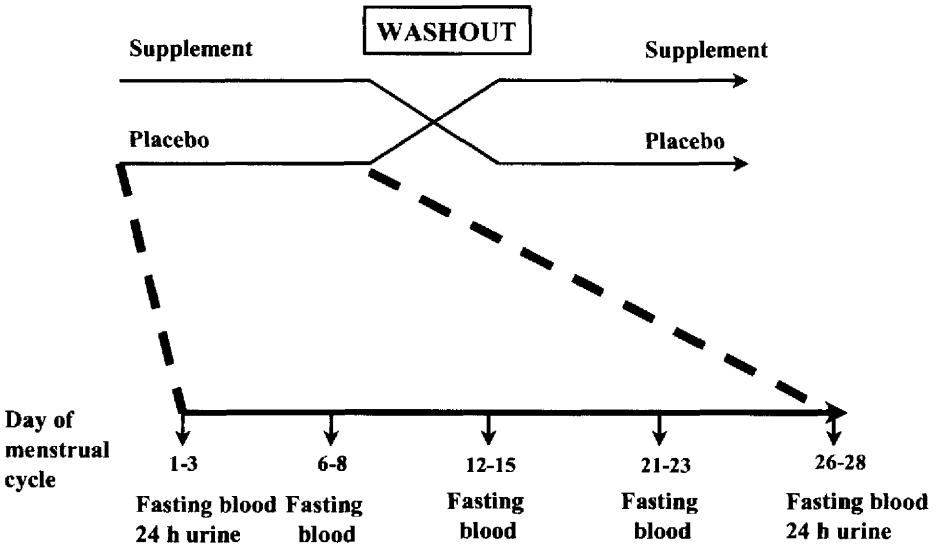


Figure 5.2.1 Study design – for pre-menopausal women blood samples were taken throughout the menstrual cycle as shown. For post-menopausal women, fasting blood and urine were only collected at the start and end of the 28 day intervention period.

5.2.2 Eligibility Criteria

Pre-menopausal - not on oral contraceptives, no antibiotics within previous 4 months, regular menstrual cycle of approximately 28 days.

Post-menopausal - not on hormone replacement therapy, no antibiotics within previous 4 months, had an oophorectomy or amenorrhoea for at least 12 months.

5.2.3 Sample Collection

Fasting blood samples were obtained from post-menopausal subjects at baseline and at 28 days in each phase of the study. Fasting blood samples

were collected at baseline, day 1-3, day 6-8, day 12-15, day 21-23 and day 26-28 from pre-menopausal subjects to assess the effects of the menstrual cycle on the endpoints measured. For all subjects, a 24-h urine sample was collected at baseline and 28 days for each phase. In addition, a fully validated food frequency questionnaire was filled in at the baseline stage of each phase and a food diary completed over the last seven days of each phase. All biological samples were stored at -80°C until analysis. The study was approved by the Royal Free and University College London Ethical Committee.

5.2.4 Endpoint measurements

A variety of endpoint measures were assessed. Levels of IGF-1, IGF-BP1, IGF-BP3 were measured in serum using ELISA methods (Diagnostic Systems Laboratories). Oestradiol, progesterone, LH, FSH, SHBG were measured in serum using routine IRMA/RIA methods. Isoflavones were assessed in urine at the start and end of each supplementation period by GC-MS according to (Morton et al., 1994). Serum lipids were assessed, again at the start and end of each supplementation phase, using standard spectrophotometric assays. Antioxidant status was assessed by the measurement of vitamins A and E and carotenoids (Craft, 1992), malondialdehyde (Young & Trimble, 1991), vitamin C (Vuillemier & Keck, 1989), and aqueous phase lipid hydroperoxides (Wolff, 1994).

Fully validated EPIC food frequency questionnaires (FFQ) and 7-day food diaries (FD) (McKeown et al., 2001) were analysed using DietPlan 5 for Windows 98.

See Chapter two for further detail on Methods.

5.2.5 Supplement

The supplement used was a proprietary product called Promensil donated by Novogen. Each 500 mg supplement tablet contained 215 mg of an extract of Trifolium pratense (red clover) leaf. This extract contained 40 mg/tablet of total isoflavones present as hydrolysed aglycones. Each tablet contained 4.0 mg genistein (4',5,7-trihydroxyisoflavone), 3.5 mg daidzein (4',7-dihydroxyisoflavone), and their methylated precursors 24.5 mg biochanin A (4'-methoxy-5,7-dihydroxyisoflavone) and 8.0mg formononetin (4'-methoxy-7-hydroxyisoflavone). The residual material in each tablet was predominantly flavonoids, principally chlorophylls a and b. Other ingredients were as follows: Calcium hydrogen phosphate, Cellulose-microcrystalline, Soy polysaccharide, Natural vitamin E, Magnesium stearate, Silica, hydroxylpropyl mehyl cellulose, Rosemary extract and iron oxide. The placebo had the same physical properties (appearance and taste) and excipient ingredients but did not contain the isoflavone extracts.

5.2.6 Statistical analyses

The intervention was double-blinded and was not un-blinded until all analyses, including statistical analyses had been completed.

Pre- and post-menopausal women were considered separately in the analyses. Changes from baseline were compared between treatment groups using a paired samples t-test (for two time-point comparisons) or repeated measures ANOVA (for multiple time-point comparisons). The association of continuous variables was assessed using Pearson correlation coefficients. For the pre-menopausal subjects, at the 5% level of significance, the study had 80% power

to detect as statistically significant a 35% difference in change in IGF-1 between the treatment groups over the supplementation period.

5.3 Results

A total of 16 pre- and 7 post-menopausal subjects completed both phases of the study. The baseline characteristics of the subjects are shown in **Table 5.3.1.** Among pre-menopausal subjects, three out of 16 were parous and among post-menopausal subjects, five out of six were parous. The mean (range) number of wash-out days was 95 (56, 206) in pre-menopausal subjects and 70 (62, 91) in post-menopausal subjects.

Table 5.3.1 Baseline characteristics of study subjects

	Pre-menopausal (n=16)	Post-menopausal (n=7)
Age	34 (9)	57 (6)
BMI	23.3 (4.3)	24.4 (5.0)
Waist to hip ratio	0.75 (0.04)	0.77 (0.05)
Protein (E%)	14.1 (2.5)	14.3 (1.6)
Carbohydrate (E%)	41.8 (6.8)	47.0 (7.6)
Fat (E%)	38.8 (4.9)	35.8 (3.8)
Alcohol (E%)	5.3 (5.1)	3.0 (3.9)

Data presented as mean (SD). E% = percentage of total calories

5.3.1 Isoflavones

The change in concentration of genistein, daidzein and equol over the supplementation period was significantly larger during the supplement phase compared to placebo in both pre- and post-menopausal women **(Table 5.3.1.1)** Concentrations excreted on the placebo were comparable to normal ranges in other studies (Cassidy, 2006). Concentrations of genistein, daidzein and equol excreted in urine, in pre-menopausal subjects, did not change during the placebo phase, but increased significantly during the supplement phase **(Fig 5.3.1.1).** This was also the case in post-menopausal subjects **(Fig 5.3.1.2).** However, the concentrations of genistein in postmenopausal subjects **(Table 5.3.1.2)** increased both on placebo (Mean±SD placebo (baseline 258.6±174.6 versus 28 days 659.2±388.5 ng/ml; $p<0.05$)) and on supplement (Mean±SD supplement baseline 749.9±374.7 ng/ml versus 28 days 3959.5±2194.4; $p<0.01$)). However, the magnitude of increase in genistein was much higher on the supplement (5.3-fold) than when on placebo (2.5-fold) and would appear to be because of a very low baseline level for genistein during the placebo phase, compared to the baseline level at the start of the supplement phase.

The placebo increase seen in post-menopausal women may have been caused by a carry over effect. Subjects who completed the supplement phase first did show higher genistein concentrations in the placebo phase nevertheless there was no statistical difference between the two groups when compared with the Mann Whitney U test (Mean±SD placebo first (placebo baseline 170.93± 65.05) and on supplement first (placebo baseline 375.6± 221.92)).

Concentrations of the two lignans, enterolactone and enterodiol, did not change during either phase, which is to be expected as neither of these compounds were present in the supplement.

The metabolite equol, was significantly elevated on the supplement though the increase varied greatly between individuals ranging from 0.0 - 4172 ng/ml. A comparison of mean urine isoflavone concentrations post-supplement with other studies, shows that equol excretion varies considerably more than the other isoflavones (between 8 and 93 fold difference) **(Table 5.3.1.3).**

Subjects with urinary equol concentrations post-isoflavone supplement of >1µg/ml were defined as equol excretors. As defined above, 23% (3/14) pre-menopausal subjects were equol excreters compared to 20% (1/5) of post-menopausal subjects. Post-supplementation, Equol excretion ranged from 0-946 ng/ml in the non-excreters to 1197-4172 ng/ml in the excreters. When the four excreters were compared to the 15 non-excreters using an independent samples t test, levels of MDA, triglycerides, γ-tocopherol and α-tocopherol (at baseline) were significantly higher in the excreters. There were no significant differences seen in the FFQ or the food diaries of these subjects. There were also no significant differences in alterations of IGF-1 and BP-3 or BP-1 concentrations in these two groups when comparing mean changes on placebo or supplement **(Table 5.3.1.4).**

Table 5.3.1.1. Effect of supplementation on urinary excretion of isoflavone metabolites in pre-menopausal subjects.

	Placebo		Supplement	
	Day 1-3	Day 26-28	Day 1-3	Day 26-28
Genistein ng/ml	254.8(212.8)	487.9(386.6)	567.2(458.2)	2756.1(2248)*
μg/day	436.5(375.3)	967.3(1064.7)	1097.4(999.5)	4641.9(3599.7)*
ng/μmol creatinine	41.6(38.2)	86.4(81.4)	106.2(93.7)	445.4(282.2)**
μmol/mol creatinine median (interquartile range)	127.2 (45.4,266.9)	186.0 (137.4, 476.5)	319.1 (39.5,731.5)	15323.1(793.7, 2254.5)**
Daidzein ng/ml	411.5(410.6)	228.5(187.5)	216.6(147.0)	3582.5(2107)**
μg/day	740.7(1032.8)	384.8(280.8)	416.9(453.5)	6362.6(5273)**
ng/μmol creatinine	64.6(79.2)	40.3(37.2)	34.9(25.2)	607.3(455.6)**
μmol/mol creatinine median (interquartile range)	147.8 (48.0, 308.7)	102.5 (63.7, 194.2)	94.0 (66.9, 231.4)	1785.7 (925.1, 3416.1)**
Equol ng/ml	94.9(108.9)	68.0(52.4)	162.2(404.6)	911.9(1133.2)
μg/day	118.4(134.1)	121.1(110.4)	167.5(286.4)	1363.4(1726)*
ng/μmol creatinine	9.22(8.8)	12.5(13.0)	16.27(28.9)	132.7(131.3)*
μmol/mol creatinine median (interquartile range)	20.8 (13.3, 56.9)	49.9 (17.8, 57.5)	32.2 (2.6, 82.8)	443.3 (152.2, 760.9)*
Enterolactoneng/ml	336.8(888.5)	198.1(274.5)	203.0(185.7)	188.3(377.8)
μg/day	542.0(1286)	332.8(474.9)	337.7(312.3)	289.0(597.7)
ng/μmol creatinine	65.1(180.7)	39.7(76.8)	32.3(30.4)	28.4(60.1)
μmol/mol creatinine median (interquartile range)	32.7 (14.4, 131.9)	66.0 (31.4, 110.7)	85.8 (24.1, 168.9)	34.9 (15.9, 86.5)
Enterodiol ng/ml	26.9(30.9)	18.4(18.3)	40.9(62.0)	18.1(18.6)
μg/day	36.7(50.4)	29.6(32.5)	74.6(137.3)	28.0(26.8)
ng/μmol creatinine	3.49(5.7)	3.14(3.54)	6.45(10.8)	2.72(2.33)
μmol/mol creatinine median (interquartile range)	5.8 (4.04, 8.87)	3.6 (1.66, 22.9)	6.17 (3.14, 19.4)	5.31 (4.25, 12.0)

Mean (SD) *$p<0.05$, **$p<0.01$ when baseline is compared with post placebo/supplement (paired t test)

Table 5.3.1.2. Effect of supplementation on urinary excretion of isoflavone metabolites in post-menopausal subjects.

	Placebo		Supplement	
	Baseline	Day 28	Baseline	Day 28
Genistein ng/ml	258.6(174.6)	659.2(388.5)*	749.9(374.7)	3959.5(2194)**
μg/day	581.0(376.4)	1362.8(886.3)*	1361.3(579.3)	8696.8(5096)**
ng/μmol creatinine	66.2(56.8)	141.0(89.4)*	131.0(76.8)	1455.5(1610)
μmol/mol creatinine median (interquartile range)	155.3 (104.0, 333.8)	617.0 (141.8, 785.9)*	411.0 (336.9, 517.9)	3538.2(1461.1,6 756.3)*
Daidzein ng/ml	343.7(196.6)	353.4(255.8)	525.5(345.4)	4964.0(3733)*
μg/day	765.4(396.8)	758.5(660.3)	1013.6(801.8)	11083(9432)*
ng/μmol creatinine	85.8(58.7)	77.8(64.6)	102.1(88.4)	1335.6(783.2)**
μmol/mol creatinine median (interquartile range)	259.4 (187.4, 496.6)	195.0 (122.2, 562.3)	301.9 (100.5, 794.2)	5717.0(2464.9,5 932.5)*
Equol ng/ml	63.0(26.7)	78.4(56.4)	86.5(39.4)	705.0(344)*
μg/day	141.6(69.6)	172.5(143.6)	163.2(72.5)	1536.9(823)*
ng/μmol creatinine	13.7(5.47)	17.7(14.2)	12.9(3.08)	256.9(267)
μmol/mol creatinine median (interquartile range)	67.2 (34.5, 73.5)	58.0 (25.5, 127.9)	50.5 (45.3, 63.2)	704.8 (282.3, 2019.6)
Enterolactoneng/ml	166.7(161.3)	142.9(74.8)	169.7(154.2)	167.0(166.5)
μg/day	364.9(334.2)	269.2(128.4)	353.3(361.5)	396.9(410.5)
ng/μmol creatinine	38.14(32.7)	27.9(13.3)	32.8(38.5)	73.7(94.0)
μmol/mol creatinine median (interquartile range)	82.2(38.2, 187.9)	108.8(74.9, 120.6)	59.5(17.2, 216.5)	119.0(21.7, 560.5)
Enterodiol ng/ml	9.95(9.08)	10.0(12.7)	8.94(5.94)	9.19(6.82)
μg/day	22.12(20.9)	22.64(31.2)	17.2(13.8)	20.2(15.4)
ng/μmol creatinine	2.51(2.54)	2.32(3.11)	1.65(1.47)	2.85(2.21)
μmol/mol creatinine median (interquartile range)	7.65(2.56, 9.01)	4.0(2.2, 11.8)	3.90(2.7, 6.25)	6.85(3.05, 18.3)

Mean (SD) *p<0.05, **p<0.01 when baseline is compared with post placebo/supplement (paired t test)

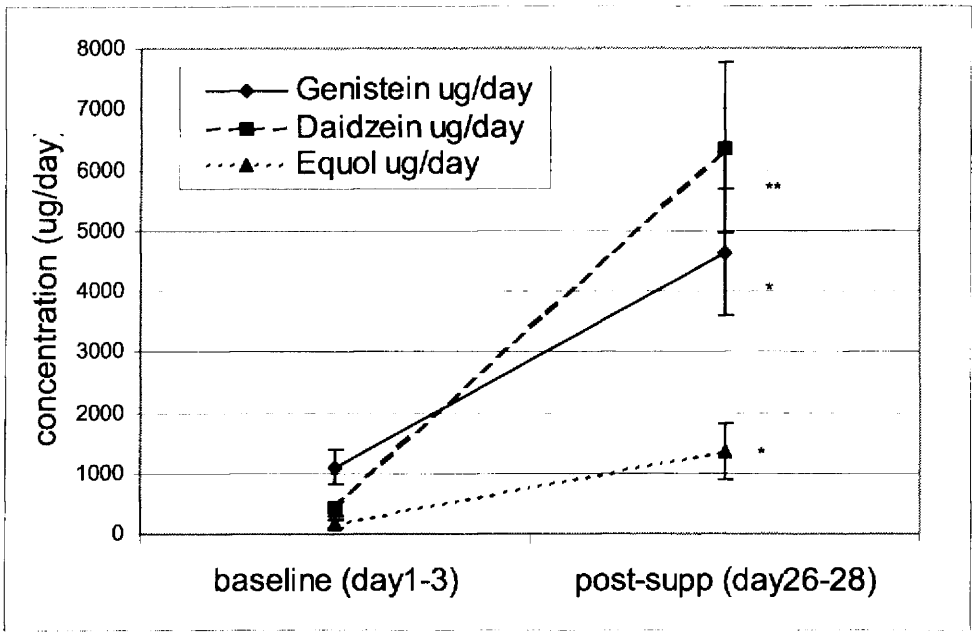


Figure 5.3.1.1 Effect of supplementation on urinary excretion of isoflavone metabolites in pre-menopausal subjects.

Mean ± SE; *p<0.05, **p<0.01 when baseline is compared with post-supplement (paired t test)

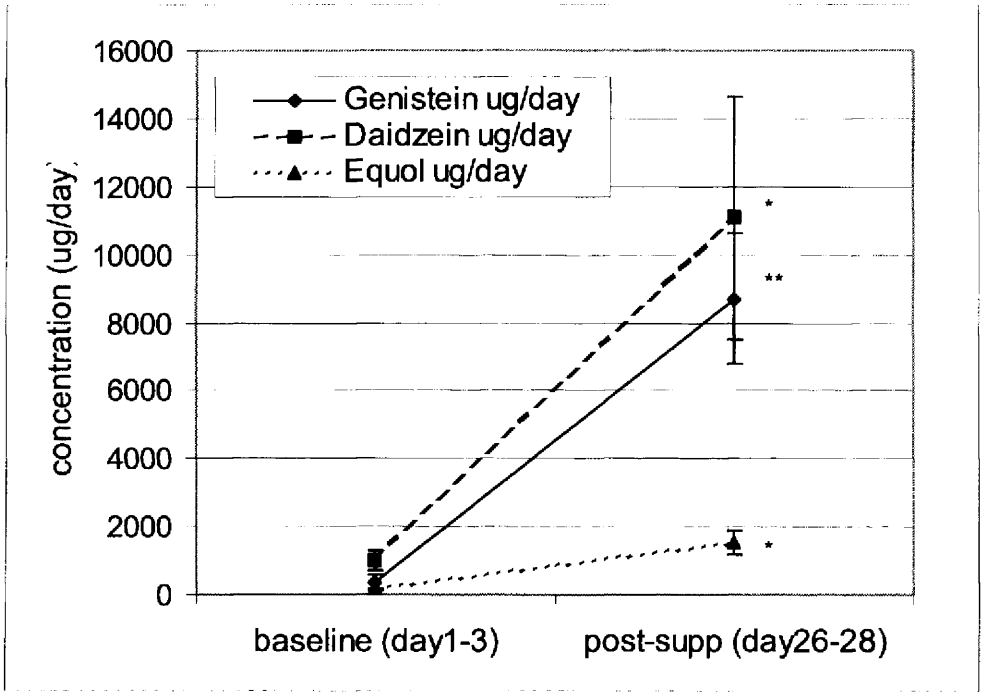


Figure 5.3.1.2. Effect of supplement on urinary excretion of isoflavone metabolites in post-menopausal subjects.

Mean ± SE *p<0.05, **p<0.01 when baseline is compared with post-supplement. (paired t test)

Table 5.3.1.3. Comparison of Isoflavone excretion with other studies

Author		Baseline	Post-supplement	Fold increase
Albertazzi (60g soy powder, 3 months)	Genistein ng/ml	114	2818	25
	Daidzein ng/ml	224	2956	13
	Equol ng/ml	42	989	23
Campbell (80mg Isoflavones, 28 days)	Genistein nmol/d	1478	20561	14
	Daidzein nmol/d	2543	26044	10
	Equol nmol/d	651	5268	8
Karr (23mg Gen, 13mg Daid in soy protein, 9 days)	Genistein nmol/d	225	5139	23
	Daidzein nmol/d	561	9303	17
	Equol nmol/d	449	5900	13
Petrakis (38mg Genistein in soy protein, 5 months)	Genistein μg/d	197	3121	16
	Daidzein μg/d	114	6007	53
Rowland (35mg Gen, 21mg Daid in soy protein, 17days)	Genistein nmol/d	384	10326	27
	Daidzein nmol/d	1060	17969	17
	Equol nmol/d	43	3995	93

Table 5.3.1.4: Comparison of serum lipids, tocopherols and IGF-1, BP-3 and BP-1 concentrations in equol excreters and non excreters.

Nutrients	Non-excreters (n=15)	Excreters (n=4)	P
MDA placebo baseline	1.85 (0.57)	4.13 (1.77)	0.000
Triglycerides placebo baseline	0.665 (0.535)	2.23 (2.13)	0.014
γ-tocopherol placebo baseline	4.00 (0.43)	5.72 (2.30)	0.009
α-tocopherol placebo baseline	21.60 (4.99)	31.44 (12.80)	0.024
IGF-1 Placebo (day 1- day 28)	-27.87 (37.93)	7.32 (19.5)	0.095
IGF-1 Supplement (day 1 – day 28)	-2.95 (49.47)	4.47 (45.51)	0.790
IGFBP-3 Placebo (day 1- day28)	-228.4(344)	-418.6 (225.8)	0.315
BP-3 Supplement (day 1-day 28)	-111.0 (334.2)	-408.2 (257.6)	0.120
IGFBP-1 Placebo (day 1-day 28)	-14.21(30.4)	-5.53 (25.3)	0.609
BP-1 Supplement (day 1-day 28)	-5.64(26.77)	-9.59 (31.02)	0.802

Mean (SD) Independent samples t test

5.3.2 Hormones

Oestradiol, progesterone, LH, FSH and SHBG all followed normal menstrual cycle patterns in pre-menopausal subjects on placebo **(Figures 5.3.2.1-5.3.2.5)**, and levels were not significantly different on the isoflavone supplement **(Table 5.3.2.1)** when compared using repeated measures ANOVA. Similarly, there was no significant difference seen between placebo and supplement hormone levels measured in post-menopausal subjects **(Table 5.3.2.2)**.

However when hormone concentrations where compared using a paried t test there was a significant increase in SHBG concentrations on day 21-23 in supplement phase vs placebo of pre-menopausal subjects (P=0.003).

When hormone levels were compared between groups, pre-menopausal women had higher levels of oestradiol (P=0.019) and Progesterone (P=0.016) and post-menopausal women had significantly higher levels of LH (P= 0.000) and FSH (P= 0.001). No significant difference was seen with SHBG levels in these groups **(Table 5.3.2.3.)**.

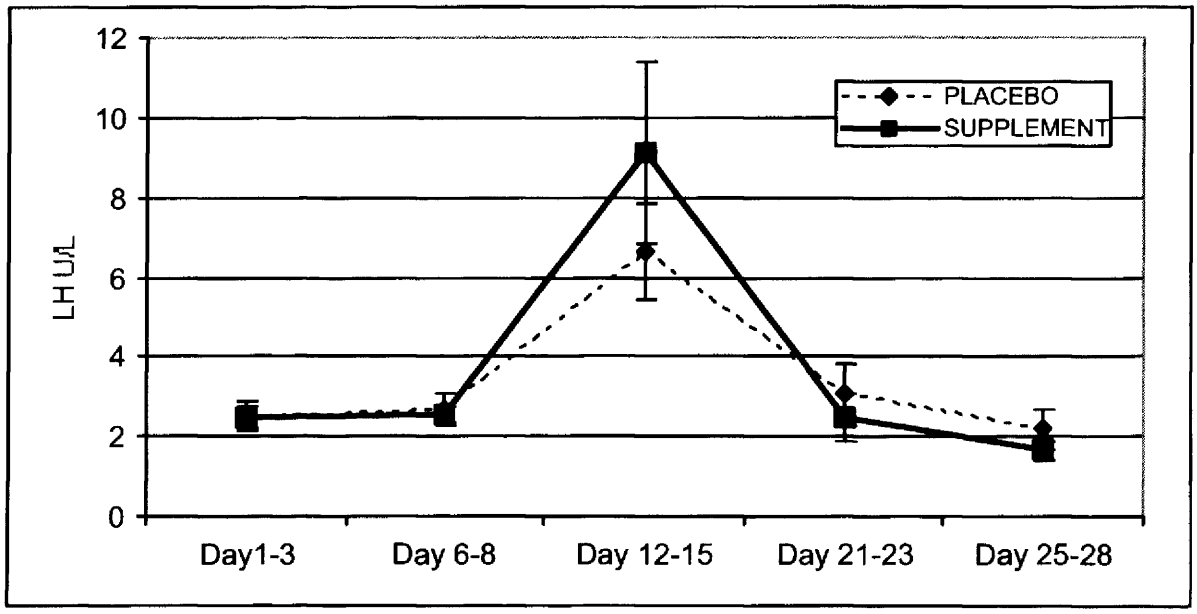


Figure 5.3.2.1: Effect of supplementation on LH levels in pre-menopausal women. Mean (SE)

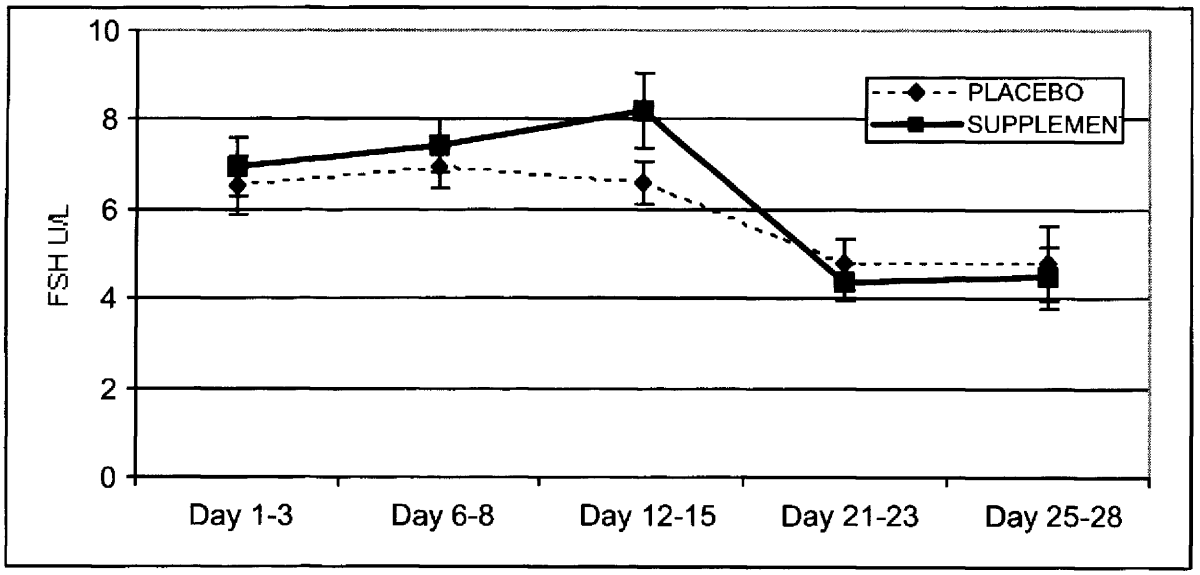


Figure 5.3.2.2: Effect of supplementation on FSH levels in pre-menopausal women. Mean (SE)

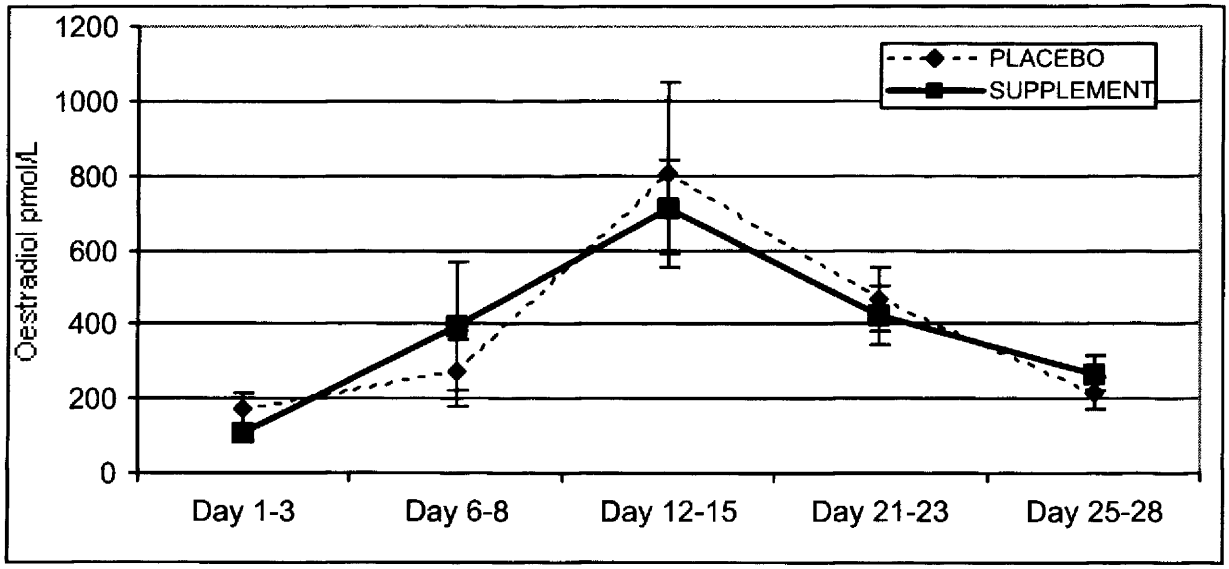


Figure 5.3.2.3: Effect of supplementation on Oestradiol levels in pre-menopausal women. Mean (SE)

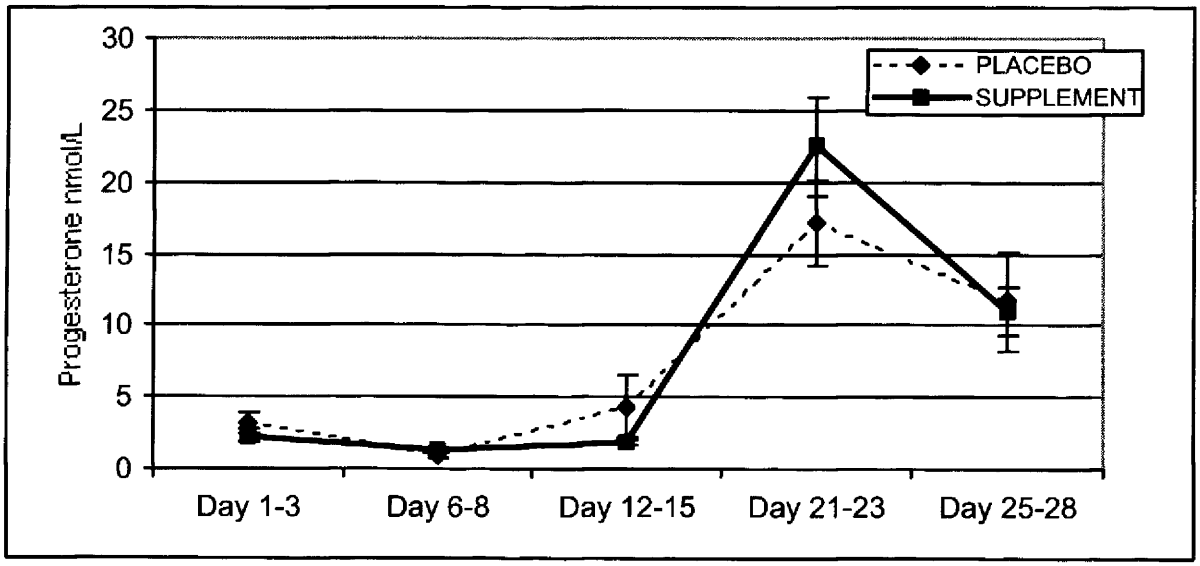


Figure 5.3.2.4: Effect of supplementation on Progesterone levels in pre-menopausal women. Mean (SE)

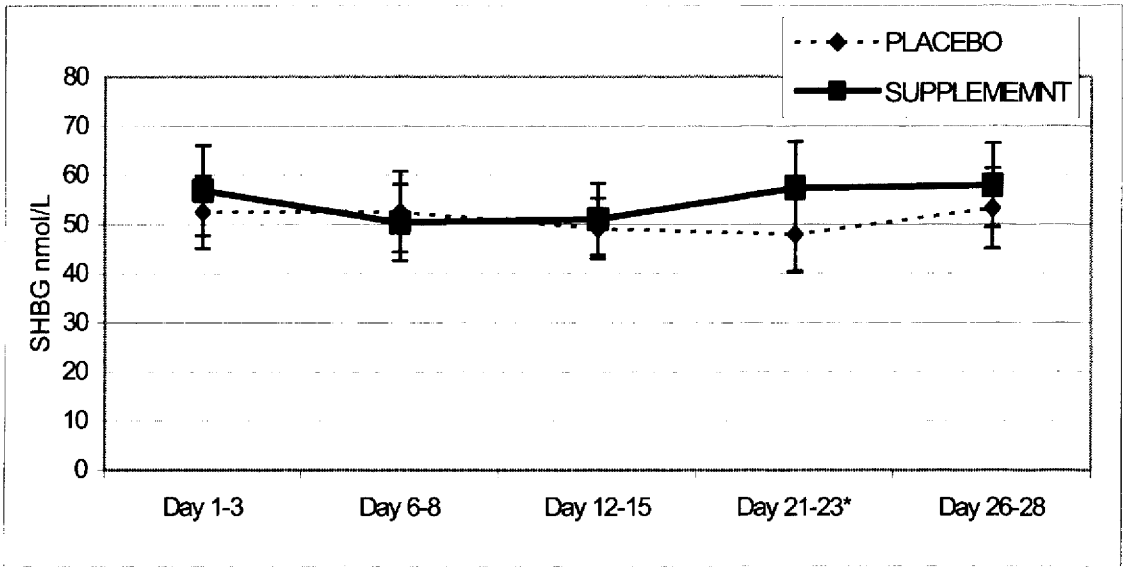


Figure 5.3.2.5: Effect of supplementation on SHBG levels in pre-menopausal women. Mean (SE). *P=0.003 paried t test supplement vs placebo (Day 21-23).

Table 5.3.2.1: Effect of isoflavone supplementation on circulating hormone levels in pre-menopausal women. Mean ± (SD).

		Baseline	Day 6-8	Day 12-14	Day 21-23	Day 26-28
LH (U/L)	Placebo	2.5 (1.0)	2.7 (1.6)	6.6 (4.6)	3.1 (3.0)	2.2 (1.9)
	Supplement	2.5 (1.4)	2.5 (0.8)	9.1 (8.8)	2.5 (2.3)	1.6 (1.0)
FSH (U/L)	Placebo	6.5 (2.6)	6.9 (1.8)	6.6 (1.9)	4.8 (2.2)	4.8 (3.2)
	Supplement	7.0 (2.5)	7.4 (2.4)	8.2 (3.2)	4.4 (1.7)	4.5 (2.6)
Oestradiol (pmol/L)	Placebo	170.7 (167.4)	271.4 (345.2)	801.7 (967.3)	463.9 (334.9)	217.1 (165.7)
	Supplement	105.5 (60.8)	396.5 (669.6)	711.9 (487.4)	425.1 (308.6)	269.1 (183.0)
Progesterone (nmol/L)	Placebo	3.2 (3.1)	1.0 (0.8)	4.3 (8.6)	17.1 (11.5)	11.6 (13.4)
	Supplement	2.3 (2.1)	1.3 (0.9)	1.9 (1.3)	22.5 (13.3)	11.0 (6.7)
SHBG (nmol/L)	Placebo	52.5 (28.2)	52.5 (31.7)	49.1 (23.7)	47.9 (28.9)	53.3 (31.7)
	Supplement	56.9 (35.3)	50.3 (30.1)	51.0 (28.3)	57.4* (36.6)	57.9 (33.0)

*P=0.003 paried t test supplement vs placebo (SHBG Day 21-23).

Table 5.3.2.2: Effect of isoflavone supplementation on circulating hormone levels in post-menopausal women. Mean ± (SD).

		Baseline	Day 28
LH (U/L)	Placebo	17.3 (4.0)	18.5 (6.5)
	Supplement	16.3 (4.3)	17.7 (6.0)
FSH (U/L)	Placebo	90.9 (39.3)	106.7 (58.6)
	Supplement	86.7 (32.8)	97.6 (48.5)
Oestradiol (pmol/L)	Placebo	49.1 (43.4)	40.1 (13.4)
	Supplement	42.1 (17.6)	47.6 (25.5)
Progesterone (nmol/L)	Placebo	1.05 (0.41)	1.09 (0.62)
	Supplement	0.91 (0.73)	0.99 (0.47)
SHBG (nmol/L)	Placebo	62.8 (35.9)	61.8 (28.4)
	Supplement	61.9 (24.5)	63.0 (24.0)

Table 5.3.2.3: Comparison of IGFs and hormone levels measured by menopausal status.

	Premenopausal (n=16) Mean (SD)	Postmenopausal (n=7) Mean (SD)	Mean Difference (95%CI)	Sig. (2-tailed)
Age (y)	33.9(8.5)	57.4 (5.7)	-23.49 (-29.87, -17.11)	0.000
IGF-1 (ng/ml)	169.6 (51.4)	125.9 (43.5)	43.68 (-1.24, 88.6)	0.056
IGFBP-1 (ng/ml)	53.4 (39.9)	47.9 (16.6)	5.51 (-19.0, 30.0)	NS
IGFBP-3 (ng/ml)	3116 (395)	2874 (418)	241.7 (-168.6, 652.0)	NS
Oestradiol (pmol/l)	170.7 (167.4)	49.1 (43.4)	113.5 (20.7, 206.4)	0.019
Progesterone (nmol/l)	3.16 (3.10)	1.05 (0.41)	2.07 (0.45, 3.70)	0.016
LH (U/l)	2.5 (1.0)	17.3 (4.0)	-14.5 (-18.2, -10.8)	0.000
FSH (U/l)	6.5 (2.6)	90.9 (39.3)	-84.1 (-120.5, -47.7)	0.001
SHBG (nmol/l)	52.5(28.2)	62.8 (35.9)	-11.0 (-45.2, 23.3)	NS

Data taken from placebo baseline levels. Comparisons done with two sample t test and equal variances not assumed.

5.3.3 Insulin-like Growth Factors

Baseline concentrations of IGF-1, BP1 and BP3 fell within the expected ranges given by the manufacturers of the ELISA kits used (Diagnostic Systems Laboratories).

No significant difference was seen in IGF levels between pre and post - menopausal women, however there were slightly higher levels of IGF-1 in pre-menopausal women at baseline (P=0.056) **(Table 5.3.2.3)**.

For pre-menopausal subjects, while there was an effect of time on IGF-1 ($p=0.005$), IGF-BP1 ($p=0.004$), and IGF-BP3 ($p<0.001$) confirming that IGF profile is influenced by menstrual cycle, this did not differ between placebo and isoflavone supplement **(Table 5.3.3.1) (Figures 5.3.3.1-5.3.3.3)**.

When the change in IGF-1 over the whole supplementation period was compared between the supplement and placebo phases, there was a non-significant reduction in change in IGF-1 ($p=0.06$) on isoflavone supplement compared to placebo. However, this may be due to the non-significantly higher baseline concentrations of IGF-1 during the supplement phase.

In post-menopausal subjects, there was no effect of isoflavone supplementation in comparison with placebo on IGF-1, IGFBP-1 or IGFBP-3 **(Table 5.3.3.2)**.

Table 5.3.3.1: Effect of supplementation on insulin-like growth factor and binding protein concentrations in pre-menopausal women

		Baseline (Day 1-3)	Day 6-8	Day 12-15	Day 21-23	Day 26-28
IGF-1 (ng/ml)	Placebo	**169.6 (51.4)**	170.3 (55.5)	159.7 (64.4)	192.1 (50.2)[ac]	194.5 (49.5)[a]
	Supplement	**193.7 (71.1)**	175.3 (55.0)	177.5 (57.1)	204.2 (46.0)[c*]	197.4 (52.1)
IGFBP-1 (ng/ml)	Placebo	**53.4 (39.9)**	49.9 (38.2)	43.3 (28.8)	46.6 (32.8)	64.4 (50.0)
	Supplement	**59.1 (44.1)**	54.5 (36.1)	63.5 (57.5)	54.5 (48.3)	72.9 (52.5)
IGFBP-3 (ng/ml)	Placebo	**3116 (395)**	3195 (425)	3237 (404)	3319 (446)[a]	3411 (419)[a*]
	Supplement	**3275 (629)**	3254 (478)	3305 (417)	3343 (446)	3497 (484)[b]

Data presented as mean (SD). Repeated measures ANOVA revealed significant changes over the menstrual cycle during placebo phase in IGF-1 ($p=0.005$), IGF-BP1 ($p=0.004$) and IGF-BP3 ($p<0.001$) but no significant differences between treatment regimen in these changes (IGF-1 $p=0.44$; IGF-BP1 $p=0.48$; IGF-BP3 $p=0.81$).

[a] *(paired t test significantly different from placebo baseline)*

[b] *(paired t test significantly different from supplement baseline)*

[c] *(paired t test significantly different from follicular phase Day 6-8)*

* *($p \leq 0.01$)*

Table 5.3.3.2: Effect of supplementation on insulin-like growth factor and binding protein concentrations in post-menopausal women

		Baseline Mean (SD)	28 d Mean (SD)	Mean change from baseline (SD)	Difference in mean change from baseline (95% CI)	*P* value
IGF-1 (ng/ml)	Placebo	125.9 (43.5)	137.3 (40.5)	11.37 (27.17)	0.49 (-36.93, 37.90)	0.98
	Supplement	130.2 (34.9)	141.1 (40.3)	10.89 (28.93)		
IGFBP-1 (ng/ml)	Placebo	47.9 (16.6)	48.7 (18.0)	0.76 (22.60)	-9.61 (-33.68, 14.46)	0.37
	Supplement	44.3 (9.0)	54.7 (10.7)[1]	10.37 (8.92)		
IGFBP-3 (ng/ml)	Placebo	2874 (418)	3005 (390)	92.7 (223.3)	-37.7 (-263.8, 188.3)	0.70
	Supplement	2878 (457)	2971 (516)	130.4 (225.6)		

[b]Mean change from baseline calculated as (28 days- baseline). Difference in mean change from baseline calculated as (placebo-supplement), and compared using a paired t-test.

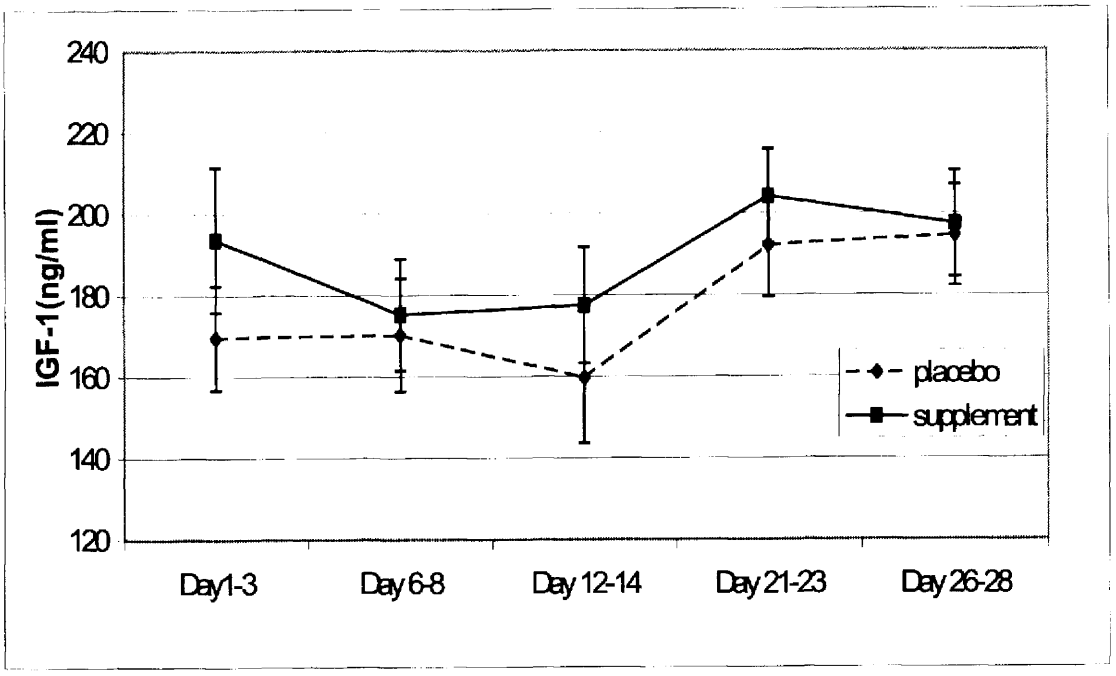


Figure 5.3.3.1: IGF-1 levels in pre-menopausal women on placebo or supplement. Mean (SE)

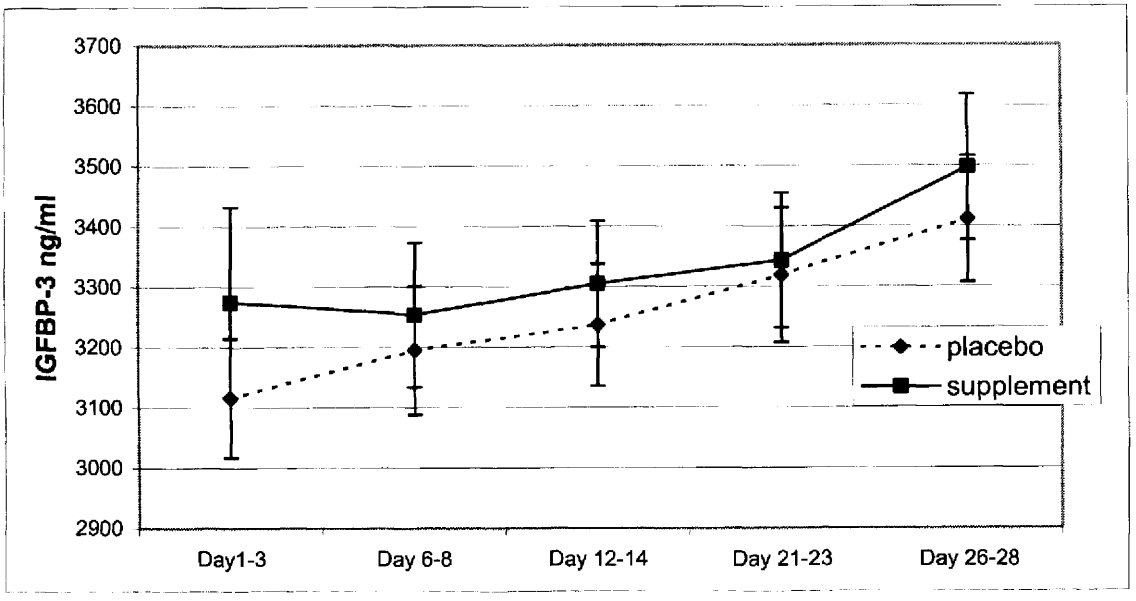


Figure 5.3.3.2: IGFBP-3 levels in pre-menopausal women on placebo or supplement. Mean(SE)

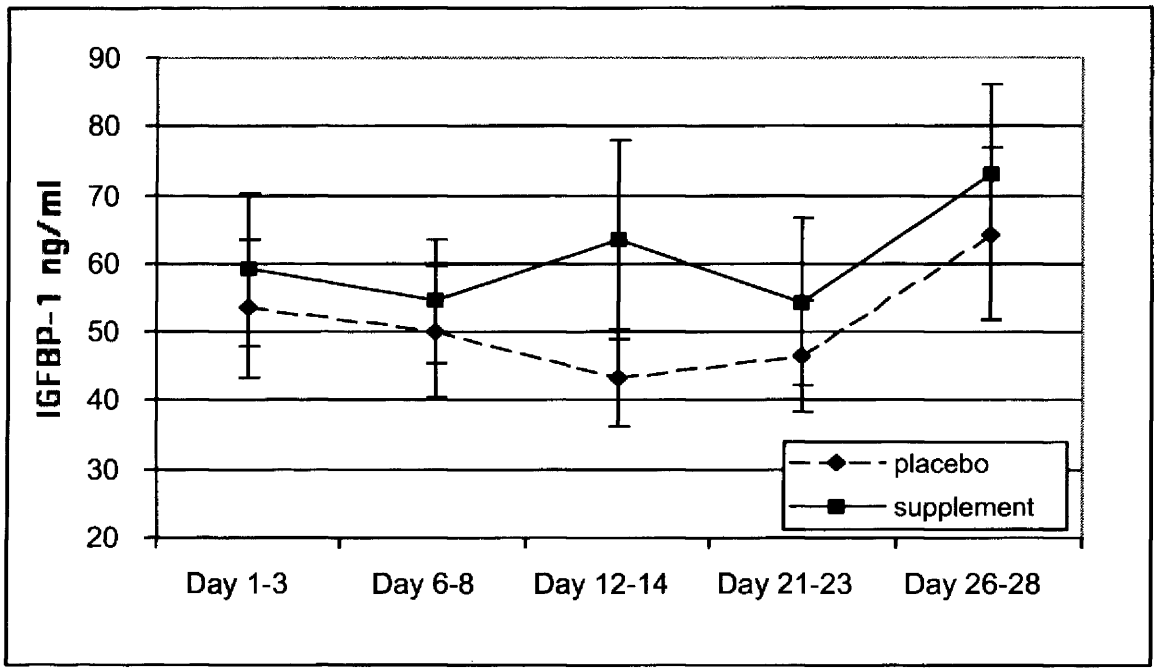


Figure 5.3.3.3: IGFBP-1 levels in pre-menopausal women on placebo or supplement. Mean (SE)

5.3.4 Lipids and antioxidants

Total cholesterol and triacylglycerol were not affected by the supplement in either pre- or post-menopausal women. However, HDL-cholesterol concentrations were significantly elevated by isoflavone supplementation in the post-menopausal subjects. **(Figure 5.3.4.1)**. There were no significant differences in any of the antioxidant endpoints assessed in either pre- or post-menopausal women when compared using paired t test **(Table 5.3.4.1)** and **(Table 5.3.4.2)**.

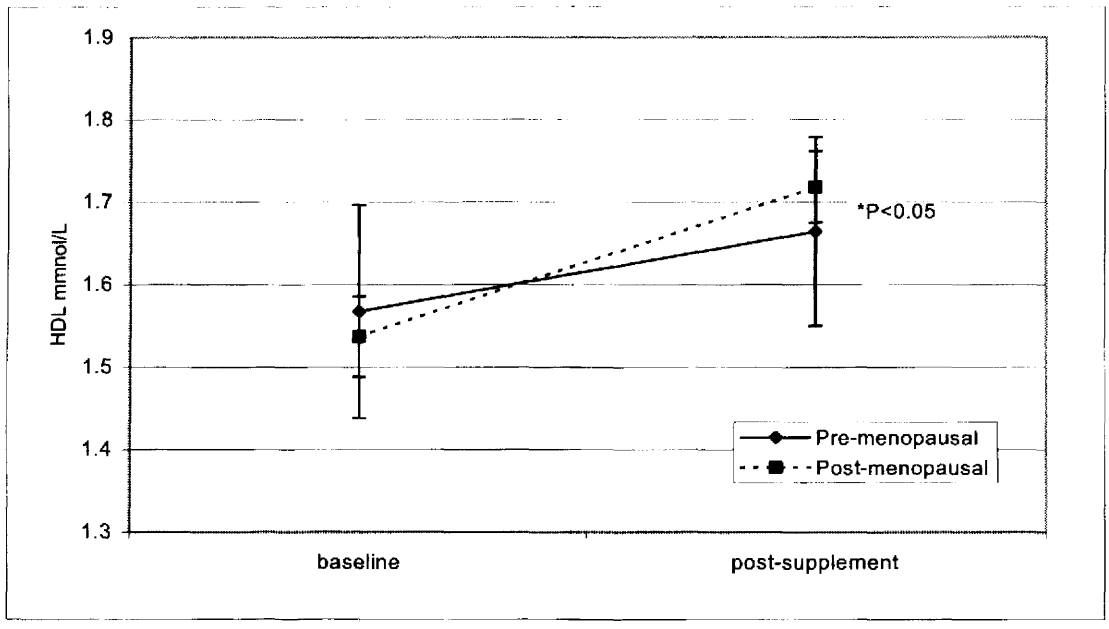


Figure 5.3.4.1:HDL-cholesterol (mmol/L) in pre and post-menopausal women after supplementation. Mean(SE).

Table 5.3.4.1: Effect of isoflavone supplementation on antioxidant and lipid status in pre-menopausal subjects

		Baseline Mean (SD)	28 d Mean (SD)	Mean change from baseline (SD)	Difference in mean change from baseline (95% CI)	*P* value
Vitamin C (μmol/l)	Placebo	93.6 (47.1)	68.7 (34.1)	-24.96 (54.24)	-26.01 (-61.43, 9.40)	0.14
	Supplement	69.3 (30.0)	70.4 (41.7)	1.05 (31.90)		
MDA (μmol/l)	Placebo	2.30 (1.08)	2.26 (0.69)	-0.04 (1.03)	-0.14 (-0.67, 0.39)	0.59
	Supplement	2.22 (0.98)	2.32 (0.74)	0.10 (0.85)		
Total cholesterol (mmol/l)	Placebo	4.65 (1.10)	4.56 (0.76)	-0.09 (0.57)	-0.09 (-0.58, 0.41)	0.71
	Supplement	4.75 (0.72)	4.75 (0.93)	0.001 (0.47)		
HDL-cholesterol (mmol/l)	Placebo	1.63 (0.58)	1.61 (0.41)	-0.03 (0.26)	-0.12 (-0.28, 0.04)	0.13
	Supplement	1.57 (0.52)	1.66 (0.46)	0.10 (0.18)		
Triacylglycerol (mmol/l)	Placebo	0.73 (0.93)	0.63 (0.31)	-0.10 (0.69)	0.01 (-0.34, 0.36)	0.96
	Supplement	0.72 (0.49)	0.62 (0.30)	-0.11 (0.30)		
LDL- cholesterol (mmol/l)	Placebo	2.68 (0.89)	2.67 (0.67)	-0.02 (0.41)	0.03 (-0.38, 0.44)	0.87
	Supplement	2.86 (0.69)	2.81 (0.92)	-0.05 (0.44)		
α-tocopherol (μmol/l)	Placebo	21.0 (7.0)	18.9 (4.1)	-2.06 (8.44)	-2.78 (-8.05, 2.49)	0.28
	Supplement	18.4 (3.3)	19.2 (3.2)	0.72 (4.35)		
γ-tocopherol (μmol/l)	Placebo	4.4 (1.4)	3.8 (0.8)	-0.56 (1.79)	-0.32 (-1.42, 0.79)	0.55
	Supplement	4.2 (1.1)	3.9 (0.6)	-0.25 (0.74)		
Retinol (μmol/l)	Placebo	2.4 (0.7)	2.1 (0.6)	-0.31 (0.74)	-0.46 (-1.06, 0.13)	0.12
	Supplement	2.2 (0.7)	2.3 (0.5)	0.16 (0.60)		
lutein	Placebo	0.32 (0.15)	0.28 (0.12)	-0.04 (0.14)	-0.05 (-0.17, 0.07)	0.38

(μmol/l)	Supplement	0.28 (0.12)	0.29 (0.12)	0.01 (0.11)		
zeaxanthin	Placebo	0.089 (0.046)	0.082 (0.035)	-0.007 (0.041)	-0.087 (-0.044, 0.027)	0.61
(μmol/l)	Supplement	0.076 (0.036)	0.078 (0.029)	0.002 (0.036)		
β-cryptoxanthin	Placebo	0.22 (0.11)	0.20 (0.13)	-0.02 (0.10)	-0.03 (-0.15, 0.09)	0.62
(μmol/l)	Supplement	0.22 (0.15)	0.23 (0.14)	0.01 (0.15)		
α-carotene	Placebo	0.083 (0.062)	0.074 (0.070)	-0.012 (0.043)	-0.022 (-0.070, 0.027)	0.36
(μmol/l)	Supplement	0.070 (0.049)	0.083 (0.063)	0.009 (0.058)		
β-carotene	Placebo	0.82 (0.60)	0.64 (0.44)	-0.18 (0.45)	-0.32 (-0.85, 0.21)	0.22
(μmol/l)	Supplement	0.60 (0.60)	0.79 (0.61)	0.15 (0.60)		
lycopene	Placebo	0.43 (0.40)	0.36 (0.39)	-0.06 (0.55)	-0.17 (-0.67, 0.33)	0.48
(μmol/l)	Supplement	0.39 (0.32)	0.49 (0.44)	0.11 (0.46)		

Mean change from baseline calculated as (28 d – baseline). Difference in mean change from baseline calculated as (placebo – supplement), and compared using a paired t-test.

Table 5.3.4.2: Effect of isoflavone supplementation on antioxidant and lipid status in post-menopausal subjects

		Baseline Mean (SD)	28 d Mean (SD)	Mean change from baseline (SD)	Difference in mean change from baseline (95% CI)	*P* value
Vitamin C (μmol/l)	Placebo	37.9 (8.9)	34.9 (14.3)	1.13 (16.41)	12.30 (-12.18,36.78)	0.25
	Supplement	46.8 (16.2)	33.9 (18.0)	-11.17 (16.77)		
MDA (μmol/l)	Placebo	2.47 (1.90)	1.98 (0.99)	-0.49 (1.55)	-0.10 (-1.64, 1.43)	0.87
	Supplement	2.35 (0.51)	1.96 (0.47)[1]	-0.39 (0.28)		
Total cholesterol (mmol/l)	Placebo	6.12 (0.96)	6.04 (1.10)	-0.08 (0.51)	-0.52 (-1.38, 0.34)	0.19
	Supplement	5.57 (1.03)	6.01 (1.14)	0.44 (1.04)		
HDL-cholesterol (mmol/l)	Placebo	1.58 (0.62)	1.54 (0.61)	-0.04 (0.10)	-0.22 (-0.40, -0.04)	0.02
	Supplement	1.54 (0.60)	1.72 (0.64)[2]	0.18 (0.22)		
Triacylglycerol (mmol/l)	Placebo	1.42 (1.35)	1.33 (1.28)	-0.09 (0.17)	-0.12 (-0.58, 0.35)	0.56
	Supplement	1.15 (0.95)	1.18 (0.87)	0.03 (0.39)		
LDL- cholesterol (mmol/l)	Placebo	3.90 (1.08)	3.90 (1.20)	-0.00 (0.42)	-0.25 (-1.05, 0.55)	0.48
	Supplement	3.52(1.12)	3.76 (1.04)	0.25 (0.94)		
α-tocopherol (μmol/l)	Placebo	28.0 (6.1)	27.4 (5.2)	-0.57 (3.48)	0.37 (-0.48, 1.21)	0.33
	Supplement	27.7 (5.0)	26.8 (4.8)	-0.94 (3.95)		
γ-tocopherol (μmol/l)	Placebo	4.39 (0.60)	4.18 (0.54)	-0.21 (0.31)	-0.03 (-0.19, 0.13)	0.69
	Supplement	4.36 (0.45)	4.18 (0.41)	0.25 (0.095)		
Retinol (μmol/l)	Placebo	2.89 (0.50)	2.74 (0.33)	-0.15 (0.45)	-0.20 (-0.66, 0.26)	0.33
	Supplement	2.79 (0.48)	2.83 (0.48)	0.04 (0.31)		
lutein	Placebo	0.27 (0.09)	0.26 (0.08)	-0.01(0.07)	-0.043 (-0.14, 0.06)	0.34

(μmol/l)	Supplement	0.29 (0.10)	0.32 (0.18)	0.03 (0.145)		
zeaxanthin	Placebo	0.08 (0.030)	0.06 (0.016)	-0.01 (0.02)	0.01 (-0.00, 0.02)	0.20
(μmol/l)	Supplement	.069 (0.033)	0.05 (0.02)	-0.02 (0.02)		
β-cryptoxanthin	Placebo	0.40 (0.29)	0.40 (0.25)	0.01 (0.08)	-0.02 (-0.10, 0.05)	0.49
(μmol/l)	Supplement	0.29 (0.12)	0.32 (0.15)	0.03 (0.06)		
α-carotene	Placebo	0.10 (0.06)	0.12 (0.08)	0.02 (0.02)	0.01 (-0.02, 0.04)	0.42
(μmol/l)	Supplement	0.09 (0.07)	0.10 (0.07)	0.01 (0.02)		
β-carotene	Placebo	0.88 (0.56)	1.03 (0.71)	0.16 (0.19)	0.029 (-0.15, 0.21)	0.71
(μmol/l)	Supplement	0.88 (0.55)	1.01 (0.67)	0.13 (0.17)		
lycopene	Placebo	0.39 (0.31)	0.39 (0.26)	0.00 (0.10)	-0.03 (-0.11, 0.06)	0.48
(μmol/l)	Supplement	0.31 (0.27)	0.34 (0.23)	0.03 (0.10)		

Mean change from baseline calculated as (28 d – baseline). Difference in mean change from baseline calculated as (placebo – supplement), and compared using a paired t-test.

5.3.5 Nutrients

When nutrient intake was assessed by food frequency questionnaire (FFQ) or 7-day food diary (FD), there was no significant difference in energy, carbohydrate, fat, protein (in grams), SFA, PUFA, MUFA, cholesterol, fibre (Southgate method), vitamin E or carotene intake between placebo or supplement phase, whether assessed by FFQ or food diary in pre- or post-menopausal subjects **(Table 5.3.5.1)**. For protein (in g) and vitamin C intake there was no significant difference between phases when assessed by FFQ. However, there was a decrease in the percentage of daily calories derived from protein in questionnaires from the supplement phase compared with the placebo phase in pre-menopausal subjects (15.0 (2.0) vs. 13.8 (1.7) placebo versus supplement, mean (SD) $p<0.05$), while vitamin C intake was lower during the supplement phase than the placebo phase in post-menopausal women when assessed by FD, (105 (41) vs. 74 (30) mg; placebo versus supplement, mean (SD) $p<0.05$). When compared with findings from the National Diet and Nutrition Survey 2000-1, percentage contribution of macronutrients to energy intake were similar to those of the national average for women (%total carbohydrate 48.5, %protein 16.6, %total fat 34.9)(Swan, 2004). Although the food diaries and FFQs showed good levels of association between assessed nutrient intake, lower levels were seen in FFQ for some micronutrients and estimated intakes of starch, energy and carbohydrate in pre-menopausal women which would suggest a tendency for under reporting. However, the FFQs appeared to give a good indication of long-term dietary habits as caloric intake was positively correlated with height (supplement, $r=0.48$, $P=0.029$), cholesterol intake with hip, weight measurements and BMI (supplement $r=0.48$, $P=0.032$) (supplement $r=0.51$, $P=0.018$) (supplement,

r=0.456, P=0.038), MUFA with hip measurements (supplement, r=0.0454, P=0.045) and percentage of fat negatively correlated with waist/hip ratio (placebo, r=-0.52, P=0.0189) (supplement, r=-0.456, P= 0.043). Intake of fibre as estimated by the Englyst method was correlated with w/hip ratio (r=0.45, P=0.047) showing that the greater the fibre intake the smaller the hips. Intake of protein correlated with waist and hip measurements in both phases; waist (placebo r=0.460, P=0.041), hip (placebo r=0.489, P=0.029), waist (supplement r=0.477, P=0.034), hip (supplement r=0.453, P=0.045).

With FDs only height correlated with caloric intake (r=0.551, P= 0.014) cholesterol (r=0.518, P= 0.023), fat (r=0.526, P=0.021) MUFA (r=0.539, P=0.017) and starch (r=0.570, P=0.011) on the supplement phase.

Table 5.3.5.1: Dietary intake, on placebo and supplement in pre and post-menopausal women. Mean (SD)

Nutrients	Unit / day		Pre-menopausal		Post-menopausal	
			FFQ	7-FD	FFQ	7-FD
Water	gm	Placebo	2266 (848)	1907 (469)[c]	2692 (657)	2093 (600)
		Supp	3240 (3361)	1729 (567)	2601 (1090)	2186 (641)
Protein	gm	Placebo	67 (17)	77 (19)	76 (20)	70 (21)
		Supp	66 (13)	69 (19)	74 (18)	67 (22)
Fat	gm	Placebo	74 (23)	96 (28)	83 (17)	78 (23)
		Supp	83 (21)	83 (22)	84 (23)	76 (33)
Carbohydrate	gm	Placebo	204 (61)	244 (57)[d]	264 (61)	239 (61)
		Supp	211 (64)	218 (50)	264 (69)	233 (61)
Energy (kcal)	Cal	Placebo	1791 (458)	2209 (485)[d]	2105 (417)	1940 (479)
		Supp	2855 (3512)	1929 (431)[c*]	2086 (372)	1898 (603)
Energy (kJ)	kJ	Placebo	7514 (1923)	9268 (2025)[d]	8835 (1758)	8173 (2018)
		Supp	11970 (14737)	8098 (1796) [c*]	8758 (1560)	8005 (2537)
Total Nitrogen	gm	Placebo	11 (3)	13 (3)	12 (3)	11 (3)
		Supp	17 (23)	11 (3)[c*]	12 (3)	11 (4)
Saturated fatty acids	gm	Placebo	25 (9)	34 (12)	30 (7)	29 (10)
		Supp	28 (8)	32 (12)	29 (9)	27 (14)
Mono-unsaturated fatty acids	gm	Placebo	26 (8)	32 (8)	30 (7)	25 (7)
		Supp	28 (7)	28 (8)	29 (9)	25 (12)
Poly-unsaturated fatty acids	gm	Placebo	15 (5)	18 (6)	16 (6)	15 (4)
		Supp	17 (9)	15 (5)	18 (7)	14 (5)
Cholesterol	mgm	Placebo	198 (59)	279 (105)	200 (64)	221 (87)
		Supp	206 (38)	271 (122)	200 (42)	197 (106)
Starch	gm	Placebo	97 (31)	135 (28)[d*]	123 (40)	124 (37)
		Supp	98 (28)	125 (29)[d]	124 (36)	121 (45)
Total Sugars	gm	Placebo	103 (52)	103 (37)	139 (34)	112 (42)
		Supp	109 (43)	90 (46)[c]	139 (56)	107 (30)
Dietary fibre, Southgate method	gm	Placebo	21 (7)	19 (4)	29 (8)	21 (5)
		Supp	21 (7)	17 (4)	30 (9)	20 (7)
Dietary fibre, Englyst method	gm	Placebo	16 (6)	14 (3)	24 (7)	16 (4)[d]
		Supp	16 (5)	12 (3)[b]	24 (9)	15 (6)
Sodium (Na)	mgm	Placebo	2166 (722)	3225 (637)[d*]	2450 (596)	2821 (742)
		Supp	2261 (611)	2821 (840)[d]	2672 (541)	2732 (976)
Potassium (K)	mgm	Placebo	3340 (1369)	3117 (543)	4045 (879)	3100 (609)
		Supp	4660 (5481)	2748 (501)[c]	3899 (1080)	3081 (895)
Calcium (Ca)	mgm	Placebo	816 (288)	992 (322)	945 (275)	912 (344)
		Supp	929 (255)[a]	861 (300)	896 (328)	874 (358)
Magnesium (Mg)	mgm	Placebo	286 (101)	297 (53)	371 (98)	294 (64)
		Supp	278 (66)	255 (48)[b]	362 (125)	284 (87)
Phosphorus (P)	mgm	Placebo	1168 (294)	1391 (291)	1377 (276)	1278 (341)
		Supp	1203 (224)	1186 (288)[b*]	1404 (376)	1241 (445)
Iron (Fe)	mgm	Placebo	10 (3)	13 (3)[d*]	12 (3)	12 (3)
		Supp	10 (2)	11 (3)[b]	12 (3)	13 (4)
Copper (Cu)	mgm	Placebo	1 (0.4)	1 (0.3)[d]	1 (0.3)	1 (0.3)
		Supp	2 (2)	1 (0.3)	1 (0.5)	1 (0.4)
Zinc (Zn)	mgm	Placebo	7 (2)	9 (2)[d]	9 (2)	8 (2)
		Supp	10 (12)	8 (2)[c]	9 (2)	8 (3)
Chloride (Cl)	mgm	Placebo	3449 (1117)	4917 (1084)[d]	3946 (943)	4371 (1116)

		Supp	5608 (8026)	4409 (1375)[c*]	4191 (826)	4181 (1455)
Manganese (Mn)	mgm	Placebo	4 (2)	3 (1)	6 (1)	4 (1)
		Supp	5 (4)	3 (1)	6(2)	4 (1)
Selenium (Se)	ugm	Placebo	45 (21)	54 (15)	63 (21)	58 (18)
		Supp	66 (98)	50 (15)	68 (18)	58 (31)
Iodine (I)	ugm	Placebo	100 (40)	110 (39)	96 (21)	111 (62)
		Supp	154 (174)	96 (31)	96 (24)	128 (66)
Retinol	ugm	Placebo	334 (213)	668 (731)[c]	531 (245)	562 (347)
		Supp	389 (182)	444 (248)	540 (216)	459 (287)[c]
Carotene	ugm	Placebo	4038 (2282)	2520 (835)[cd]	3924 (1494)	2157 (1037)[d]
		Supp	4314 (2429)	2749 (1212)[d]	3586 (1007)	2150 (1746)
Vitamin D	ugm	Placebo	2 (1)	2 (2)	2 (1)	3 (1)
		Supp	3 (4)	2 (1)	3 (1)	3 (1)
Vitamin E	mgm	Placebo	9 (3)	9 (3)	11 (6)	9 (3)
		Supp	12 (10)	7 (2)	12 (6)	9 (2)
Thiamin	mgm	Placebo	1 (0.4)	2 (3)	2 (0.5)	2 (0.4)
		Supp	1 (0.3)	1 (0.4)	2 (1)	3 (3)
Riboflavin	mgm	Placebo	2 (0.5)	2 (1)	2 (1)	2 (1)
		Supp	2 (0.4)	2 (0.4)[b]	2 (1)	2 (1)
Niacin	mgm	Placebo	21 (6)	19 (4)	25 (7)	20 (5)
		Supp	19 (5)[a]	16 (5)[bc*d]	27 (11)	18 (6)
Tryptophan divided by 60	mgm	Placebo	14 (4)	16 (4)	15 (4)	15 (4)
		Supp	14 (3)	15 (4)	14 (4)	14 (5)
Vitamin B6	mgm	Placebo	2 (1)	2 (0.5)	2 (1)	2 (1)
		Supp	2 (0.4)	2 (0.4)[d]	2 (0.4)	2 (1)
Vitamin B12	ugm	Placebo	2 (1)	3 (1)[d*]	2 (1)	3 (2)
		Supp	2 (1)	3 (1)[d]	2 (1)	4 (4)
Folate	ugm	Placebo	354 (129)	268 (84)[d]	393 (123)	266 (83)
		Supp	332 (73)	230 (68)[d]	408 (154)	276 (111)
Pantothenate	mgm	Placebo	4 (1)	4 (1)	6 (1)	4.4 (1.1)
		Supp	4 (1)	4 (1)	6 (1)	3.9(1.4)[b]
Biotin	ugm	Placebo	35 (11)	35 (9)[c]	46 (15)	37 (11)
		Supp	35 (7)	31 (9)	46 (19)	32 (8)
Vitamin C	mgm	Placebo	192 (123)	94 (36)[c*d*]	231 (85)	105 (41)[d*]
		Supp	168 (69)	93 (53)[d]	232 (99)	74 (30)[bd*]
% Calories from						
Protein		Placebo	15 (2.0)	14 (2)	14 (2)	14 (2)[c]
		Supp	13.8 (1.7)[a]	14 (2)	14 (3)	14 (2)
Carbohydrate		Placebo	43 (6)	42 (7)[c]	47 (5)	47 (8)
		Supp	41 (6)	43 (6)	47 (8)	47 (5)[c]
Fat		Placebo	37 (6)	39 (5)	36 (4)	36 (4)
		Supp	3 (4)	38 (5)	36 (7)	35 (5)[c*]
Alcohol		Placebo	5 (6)	5 (5)[c*]	3 (3)	3 (4)[c*]
		Supp	7 (8)	5 (4)[c*]	2 (2)	4 (4)[c*]

a : mean values of FFQ on supplement significantly different from FFQ on placebo (P<0.05 paired t test)
b : mean values of FD on supplement significantly different from FDs on placebo (P<0.05 paired t test)
c : mean values of FFQ significantly correlated with FD on placebo and supplement. (P<0.05 Pearsons correlation)
d: mean values of FFQ significantly different from FD (P<0.05 paired t test)
*: P<0.01

5.3.6 Associations between continuous variables

This study was a randomised, placebo-controlled intervention study, designed to determine the effect of isoflavone supplementation on a number of endpoints. A comparison of changes in the endpoints of interest between intervention and control groups therefore represents the main analysis of this study. However, secondary analysis, which due to the small numbers, multiple comparisons, and study design must be considered only as hypothesis-generating, has been carried out to assess cross-sectional associations between variables. These are presented as Tables in Appendix, and results that are consistent across more than one timepoint or method of assessment are presented here.

5.3.6.1 Associations with isoflavone status

When Isoflavone excretion in µg/day was compared with serum lipid/antioxidant levels, dietary intake and anthropometric measurements **(Tables 5.A.1 – 5.A.8)**, the following associations were found:

Baseline levels of **equol** excretion correlated positively with energy intake at baseline, assessed by both FFQ ($p=0.019$) and FD ($p=0.022$). This may have largely been due to alcohol intake, as there was also a strong positive correlation with alcohol consumption and equol excretion at baseline on both placebo and supplement of the FFQ ($p<0.001$ and $p<0.001$ respectively) and FD ($p=0.037$ and $p=0.001$ respectively).

Excretion of **genistein** (ug/day) at baseline correlated with serum levels of a number of antioxidants including retinol ($p=0.025$), α-carotene ($p=0.046$), lutein ($p=0.011$) and β-cryptoxanthin ($p=0.034$). Genistein at baseline also correlated with estimated intakes of carotene on placebo FFQ ($p=0.020$) **(Table 5.A.4)**, and FD vitamin C ($p=0.023$), suggesting overall an association between genistein status and antioxidant intake and status. However, as presented

earlier, there was no direct effect of isoflavone supplementation, leading to increased genistein excretion, on antioxidant status.

When isoflavone excretion in µg/day in pre-menopausal women was compared with serum levels of IGF-1, BP3 and BP1, there were strong positive associations between enterolactone and BP-1 throughout the cycle on both placebo and supplement **(Table 5.A.7)**.

In post-menopausal women there was a strong positive correlation between levels of daidzein post supplement and levels of IGF-1 post-supplement ($p=0.004$), although this was not seen at other timepoints. Enterolactone at baseline was positively associated with IGF-1 both on placebo ($p=0.029$) and supplement ($p=0.034$) **(Table 5.A.8).** These observations suggest some association between status of the lignan enterolactone and the IGF system.

5.3.6.2 Associations with hormonal status

When hormone concentrations were compared with isoflavone excretion in µg/day (Tables 4.A.9-4.A.10) the following associations were found:

In pre-menopausal women **(Table 5.A.10)** baseline levels of **equol** were negatively associated with levels of SHBG throughout the cycle on placebo and on days 1-3, 21-23 and 26-28 of the supplement phase.

Daidzein excretion at baseline, was positively correlated with concentrations of LH, particularly in the follicular ($p<0.001$) and luteal ($p<0.001$) phase of the cycle, and with FSH concentrations throughout the cycle on both placebo and supplement. Baseline excretion of **genistein** was also positively correlated with FSH and LH throughout the cycle.

In post-menopausal women **(Table 5.A.9)** baseline levels of **genistein** excretion correlated negatively with SHBG and FSH levels at baseline whilst on supplement.

Again, however, we saw no effect of supplementation with these compounds on hormonal status.

5.3.6.3 Associations with IGF-1 and BPs

The associations between IGF-1 and binding proteins and hormonal status are presented in Tables **5.A.11-5.A.13**. When pre-menopausal hormone levels were compared with IGF-1 and BP levels **(Table 5.A.11)** there were significant negative correlations between IGF-1 and oestradiol in the Luteal phase (day 21-23) both on placebo and supplement. There were also strong positive correlations between progesterone and BP-1 at baseline and mid cycle. SHBG levels were also significantly positively correlated to BP-1 levels throughout the menstrual cycle with some weaker negative correlations with IGF-1 at baseline **(Table 5.A.12)**.

In post-menopausal subjects, there were some correlations with hormone levels **(Table 5.A.13)** with SHBG showing positive correlations with IGF-1 levels, which were not seen in pre-menopausal women.

Correlations between IGF-1, BP-3 and BP-1 levels and Isoflavone concentrations are found in **Table 5.A.6 - 5.A.8.** Correlations between IGF-1, BP1 and BP3 and serum lipids and serum antioxidants can be found in **Tables 5.A.14 - 5.A.17** for premenopausal subjects and **Tables 5.A.19 - 5.A.20** for post-menopausal subjects. The following results are the strongest associations seen in pre and post-menopausal women.

BP-1 was positively associated with HDL on both placebo and supplement in pre-menopausal women.

In pre-menopausal women correlations with concentrations of BP-1 and BP-3 and serum antioxidants were as follows: BP-1 was associated with levels of α-

carotene (r=0.606, p=0.013, n=16) and β-Carotene (r=0.580, p=0.018, n=16) at supplement baseline.

Correlations between concentrations of IGF-1, BP1 and BP3 and dietary intake assessed by FFQ/FD can be found in **Tables 5.A.23-5.A.27.** The following correlations were of note:

In pre-menopausal women BP1 concentrations showed positive correlation with vitamin E intake throughout when assessed by FD, while in post-menopausal women there was a positive association between BP3 and BP1 with vitamin E intake, although this was not consistent at all timepoints, and BP1 and BP3 were not associated with vitamin E status.

5.3.6.4 Associations with lipid and antioxidant status

Pearson correlation coefficients comparing serum lipid levels with the intake of fat in FDs and FQs **(Table 5.A.21)** showed some significant correlations with serum levels of LDL, cholesterol and MDA with estimated intakes of Fat, PUFA, MUFA, cholesterol and % of calories from fat assessed using the FFQ in the placebo phase, although this was not shown for dietary data from food diaries.

Pearson correlations comparing serum concentrations of antioxidants with FFQ/FD intake **(Table 5.A.22)** showed strong correlations between serum levels of carotenoids and carotene intake when assessed by both FFQ and FD but especially strong correlations seen when comparing levels of α- and β-carotene with FD estimates of carotene.

5.4 Discussion

5.4.1 Isoflavones

Supplementation for 28 days with 80 mg/day red clover isoflavones significantly increased the excretion of isoflavones although levels were highly variable between individuals. As equol is a product of intestinal bacterial metabolism formed from its isoflavone precursors daidzin and daidzein (Coward et al., 1993; Setchell et al., 2002), the large range of equol's excretion after supplementation (0-27587 nmol/day) may be explained by individual differences in isoflavone metabolic pathways (Rowland et al., 2003). Feeding studies would suggest that ~30-40% of the western population excrete equol in urine when challenged with soy foods (Karr et al., 1997; Kelly et al., 1995; Rowland et al., 2000); (Lampe et al., 1998). *In vitro* and animal *in vivo* studies show equol to be more estrogenic and have greater antioxidant activity than other isoflavones (Kostelac et al., 2003; Setchell et al., 2002), with recent clinical studies finding greater benefits for equol producers, in the areas of lipid profiles, bone density, hormone profiles and IGF-1 profiles (Duncan et al., 2000; Lydeking-Olsen et al., 2004; Meyer et al., 2004; Vrieling et al., 2007; Wu et al., 2006) decreased risk of prostate cancer (Akaza et al., 2004) and inhibition of prostate cancer in animals (Lund et al., 2004).

As the proportion of metabolites excreted may be a result of differential absorption and variation in gut microflora (Atkinson et al., 2004), the influence of habitual dietary intake on excretion of equol has been a subject of considerable interest.

5.4.2 Equol metabolism and diet

The dietary habits that promote equol production still remain unclear. Animal studies have showen that the inability of some subjects to produce equol is a consequence of the lack of specific components of the gut microflora (Bowey et al., 2003). Some studies have suggested that high fibre and low fat diets are conducive to equol production. In a study which classified 30 men and women into "excreters or non- excreters" (Lampe et al., 1998), there was no difference in fat intake but rather increased calories from carbohydrate and greater amounts of plant protein and dietary fibre in the diet of excreters. Another found that equol excreters consumed significantly less fat and more carbohydrate (Rowland et al., 2000).

In theory, this type of diet may promote the growth or activity of certain strains of bacteria shown to convert Daidzein from Dihydrodaidzein (DHD) to equol *in vitro* (Decroos et al., 2005; Ueno, 2001). However, one month supplementation with 16g fibre daily in the form of wheat bran in pre-menopausal women did not increase Equol production (Lampe et al., 2001) and a recent study found that chornic soy consumption does not induce major changes to gut metabolism of isoflavones, suggesting that the bacteria and enzymes responsible for equol or O-DMA production are not inducible (Wiseman et al., 2004). Other results suggest that high fiber intake could actually decrease bioavailability of isoflavones (Tew et al., 1996). Some feeding studies done with soy milk in a metabolic unit have suggested that the consumption of soy itself can induce equol production (Lu & Anderson, 1998; Lu et al., 1996b). However, this finding was not seen in a more recent study of one month exposure to 100mg isoflavones in the form of cereal bars and yoghurts in post-menopausal women (Vedrine et al., 2006).

In this study, we did not see consistent associations between fibre intake and equol excretion, although it was not originally designed to examine this. We did find significantly higher levels of MDA, triglycerides and α- and γ-tocopherol levels in equol producers compared to non producers, and consistent associations between equol excretion and energy intake. We also found some associations between equol excretion and levels of total cholesterol ($p=0.0090$), triglycerides ($p=0.0012$), LDL ($p=0.034$), MDA ($p=0.0015$) concentrations and the fat soluble vitamins α- tocopherol ($p=0.0039$), γ-tocopherol (1.07×10^{-6}) and retinol ($p=0.034$), but this was not consistent at all timepoints and therefore these observations are probably artefactual.

Although a study comparing soy intake assessed through FFQ in 478 post-menopausal Japanese women, found no significant differences in serum lipids between soy intake groups (Somekawa et al., 2001), other studies have not compared equol excretion with serum lipid levels. However, some studies have found correlations between fat intake as assessed through dietary questionnaires and equol excretion. A study of 19 Japanese men and women consuming their traditional diet (Adlercreutz et al., 1991) reported that equol excretion correlated positively with intake of total fat ($p<0.01$), fat-fiber ratio ($p<0.05$) and meat ($p<0.05$) which was not the case with other isoflavonoids. They proposed that the individuals consuming more meat and fat had intestinal flora that metabolised daidzein into equol more readily. Two other more recent and larger studies (Hedlund et al., 2005; Ozasa et al., 2005) have also shown associations with fat intake. A study in 45 caucasian men (Hedlund et al., 2005) showed that those who had consumed ≥30mg soy for at least 2 years and consumed animal meat regularly had a higher probability of producing equol, while a study of western diets by (Lampe et al., 1999) also found isoflavone and

in particular equol excretion had strong associations with high fat and processed meat intake, and found no associations between equol excretion and soy intake. Associations examined in a Japanese population (Ozasa et al., 2005) found equol concentrations were not associated with dietary intake of tofu or miso soup (like genistein and daidzein) but with foods containing fat (butter, margarine, cheese), meat (including pork, ham) and coffee.

However, equol is actually being consumed directly from processed meats that contain soy products (Fletcher, 2003; Murphy et al., 1999) and possibly indirectly through the meat and dairy of animals consuming alfalfa, clover, or soy, supplemented feed (King et al., 1998; Lampe et al., 1999; Nilsson et al., 1967)

Certainly, within the UK population, the main contributers of isoflavones in the non-soy consuming population, were found to be bread/ bread rolls, meat products and then vegetable dishes (Mulligan et al., 2006). There is limited data on Isoflavone content of meat, milk, dairy products and fish products (King et al., 1998), (Antignac et al., 2003; Clarke et al., 2004). However, recent analysis of UK foods has found isoflavones in beers (traces of daidzein, genistein and biochanin A), fish and fish products including Indian foods (Clarke et al., 2004), with a single portion of vindaloo curry containg 11mg of genistein. Certainly, we did find strong consistent positive correlations between excretion of equol and alcohol consumption.

Analysis of Australian milk samples (King et al., 1998) found that, in areas where clover represents a significant potential source of isoflavonoids in cows milk, samples contain equol concentrations of 293± 52 ng/ml compared with 90ng/ml in Finish milk (Adlercreutz, 1985). Although the authors concluded these amounts were not clinically significant (Cassidy et al., 1994) large

quantities of milk and dairy products could account for baseline levels of equol excreted in the non soy consuming population and certainly free equol has a far greater binding affinity for SHBG (Martin et al., 1996) and oestrogen receptors alpha and beta (Muthyala et al., 2004) than its precursor daidzein.

Enterodiol and enterolactone are metabolites of plant lignans and their excretion has been associated with higher levels of dietary fibre intake (Adlercreutz, 2002a). Adlercreutz showed differences in lignan excretion, with vegetarians excreting significantly higher amounts than omnivores (Adlercreutz et al., 1986; Adlercreutz et al., 1995b). Lignan excretion in the western diet is also related to fruit intakes of squash, melons, stone fruits and berries (Hutchins et al., 1995; Lampe et al., 1999). However, we were unable to show consistent associations between fibre intake and lignan excretion in this study.

5.4.3 Isoflavones and hormones

It has been proposed that isoflavones exert their cancer preventative effects in part as a result of hormonal actions. To investigate the influence of the isoflavone supplement on regulation of the menstrual cycle and hormonal status, serum concentrations of oestradiol, progesterone, LH, FSH and SHBG were measured in all 16 pre and 7 post-menopausal women.

Hormones in pre-menopausal women followed normal menstrual cycle patterns and serum concentrations were within expected ranges for both age groups, given within kit instruction manuals and laboratory normal ranges. Analysis of pre-menopausal hormones (paired t test) showed SHBG concentrations were higher in the luteal phase on supplement compared with the luteal phase on placebo ($p=0.003$). However using repeated measure ANOVA (for multiple time point comparisons), Isoflavone supplementation had no effect on circulating

hormone concentrations in either pre- or post-menopausal subjects. Other intervention studies that have looked at the hormonal effects of soy have had varying results (Kurzer, 2002) **(Table 1.2.4.1)**. For oestradiol concentrations, some have shown increases in the follicular phase (Cassidy et al., 1994), (Duncan et al., 1999a; Kumar et al., 2002) and overall increases throughout (Petrakis et al., 1996) while others have seen decreases in the luteal phase (Lu et al., 2000a; Lu et al., 1996a; Maskarinec et al., 2002; Wu et al., 2000) and follicular phase (Lu et al., 2000a; Lu et al., 1996a; Martini et al., 1999; Nagata et al., 1998) (Nicholls et al., 2002). Only two studies have shown a significant reduction in oestradiol (Lu et al., 1996a; Wu et al., 2000). All of these mentioned studies showed a reduction in total progesterone or concentrations measured in the luteal phase but only one found this to be a significant reduction (Lu et al., 1996a). SHBG levels measured in these intervention studies also varied with only two studies showing increases in the follicular and luteal phase (Nagata et al., 1998; Wu et al., 2000). A smaller number of intervention studies have looked at the effects of soy/isofalvones on FSH and LH levels. Studies done in a metabolic unit on six pre-menopausal women (Cassidy et al., 1995; Cassidy et al., 1994) found significantly decreased FSH and LH levels in response to 45 mg isoflavones (in the form of a textured soybean product) daily, while increasing the follicular phase length of the menstrual cycle. However, in another study, 36 oz of soy milk had no effect on LH or FSH levels in 10 pre-menopausal women with no change in mean menstrual cycle length (Lu et al., 2000a), (Lu et al., 2001). Another study (Maskarinec et al., 2002) saw no significant changes in hormone levels when comparing 17 premenopausal women on 100mg soy isoflavones for a year compared to the same size control group. Studies testing three different doses of soy protein powder in 14 pre-

menopausal women (Duncan et al., 1999a; Duncan et al., 2000), found that only the low Isoflavone dose significantly decreased LH and FSH levels midcycle. A cross-sectional study by (Verkasalo et al., 2001) looked at 636 pre-menopausal women on the EPIC study who were consuming soy milk and separated them into a <142ml or >284ml vs control group. Although FSH and LH concentrations were ~25% higher in <142ml group, they found no statistically significant associations of hormones with soy milk intake in pre or post-menopausal women. When subjects were stratified into equol excreters (n=5) vs non excreters (n=9), they found that over all three different isoflavone doses, excreters had lower levels of oestrone and higher concentrations of SHBG and midluteal progesterone (Duncan et al., 2000). (Setchell et al., 2002) has hypothesised that the effects of soy may lie with an individuals ability to metabolise daidzein to the more estrogenic metabolite equol, but (Maskarinec et al., 2002) did not find a difference in oestrone or oestradiol concentrations in equol excreters after 6 and 12 months of 100mg soy isoflavone intake. Intervention with lactobacillus and birfidobacterium (Bonorden et al., 2004) was also unable to verify this hypothesis as there was no significant difference in homone concentrations in equol excreters vs non excreters with no significant alteration in equol or hormone concentrations after 2 months probiotic intervention. In a more recent randomised intervention study with 220 pre-menopausal women (Maskarinec et al., 2004), there appeared to be no significant difference in the oestrone, oestradiol, prgesterone and SHBG levels of the intervention group consuming ~50mg isoflavones daily versus the control group in samples taken in the luteal phase (day 19) after 3, 6,12 and 24 months. The author concluded that the effects of soy on the breast, if they exist,

are mediated by mechanisms other than the lowering of circulating oestrogen levels in pre-menopausal women.

A one-month crossover intervention study using soy milk (113-202 mg/day isoflavone content) or an isoflavone-free soymilk (<4.5 mg/day isoflavones), with a 4-month washout between phases, looked at the effect on the metabolism of 17β-oestradiol (Lu et al., 2000b). Higher levels of two putative carcinogenic metabolites of I7β-oestradiol, 4- and 16α-hydroxy oestrogen, and lower amounts of anti-carcinogenic metabolites, 2-hydroxyoestrogens, have been associated with a greater risk of breast cancer. The diet rich in isoflavones increased the cycle mean daily urinary excretion of 2-hydroxyestrone, while the mean daily excretion of 16α-hydroxy-estrone did not change. The ratio of 2-hydroxyestrone to 16α-hydroxy-estrone was higher during the isoflavone-rich diet (2.6 $\pm$ 0.34) than during the isoflavone-free diet (2.0 $\pm$ 0.32; p=0.01). These results suggest that isoflavones increase the metabolism of endogenous oestrogens to the protective 2-hydroxylated oestrogens in women, and this may play a role in lowering 17β-oestradiol levels and therefore reducing breast cancer risk (Lu et al., 2000b).

In post-menopausal women most soy intervention studies have shown few differences in levels of oestradiol, LH, or FSH **(Table 1.2.4.2)** (Baird et al., 1995; Berrino et al., 2001; Brooks et al., 2004; Brzezinski, 1997; Duncan et al., 1999b; Foth & Nawroth, 2003; Murkies et al., 1995; Nicholls et al., 2002; Persky et al., 2002; Petrakis et al., 1996; Pino et al., 2000). In the post-menopausal arm of a study with three different doses of soy protein, there was a small decrease in oestrone and oestradiol concentrations with a significant increase in SHBG on the high isoflavone diet containing ~132mg/per day for 93 days (Duncan et al., 1999b). Other studies have also seen an increase in SHBG (Berrino et al.,

2001; Kaaks et al., 2003; Pino et al., 2000). A study containing large quantities of isoflavones (Brzezinski, 1997) saw an increase in SHBG and a decrease in the strength of hot flushes, while (Nicholls et al., 2002) saw a decrease in LH peaks after discontinuation of soy containing 60mg isoflavones suggesting a residual effect post supplementation. In the large EPIC –Oxford cross-sectional study (Verkasalo et al., 2001), there were no differences seen in oestradiol or SHBG levels in 456 postmenopausal women consuming daily soy milk. Another arm of the EPIC study (Norfolk) found that urinary and serum levels of daidzein and genistein were negatively correlated with plasma oestradiol (Low et al., 2005), but that when women with certain genotypes were excluded these correlations were no longer significant. The author concluded that polymorphisms in genes encoding for enzymes involved in oestradiol metabolism (i.e, ESR1 PvuII CC genotype) may affect the concentration of plasma oestradiol, suggesting diet-gene interactions.

A study looking at correlations between levels of soy intake (assessed through FFQ) in 50 pre-menopausal Japanese women found negative correlations with oestradiol levels on day 11 and 22 of the cycle and no associations with SHBG (Nagata et al., 1997). Positive correlations were found between urinary phytoestrogens and plasma SHBG in other studies (Adlercreutz et al., 1987; Adlercreutz et al., 1992). More recent cross sectional studies of post-menopausal women (Low et al., 2005; Shoff et al., 1998; Thomas et al., 1999; Verkasalo et al., 2001) assessed phytoestrogen intakes through dietary questionnaires and found correlations between intake and SHBG or oestradiol were not significant.

Certainly in this study we did not see any significant change in hormone concentration in pre or post-menopausal women when on supplement.

However, the almost significant luteal increase in SHBG has been seen in other studies (Duncan et al., 2000; Wu et al., 2000). We saw no consistent association of lignan status with SHBG, in contrast with(Adlercreutz et al., 1987), who found that intake of total fibre correlated with enterolactone, enterodiol and plasma SHBG.

Further investigations are needed to determine whether isoflavones can cause hormonal changes and whether these are length of intervention and dose dependant. However data from this and other short term feeding studies suggest that Isoflavone effects on serum hormones and the menstrual cycle are unlikely to be the primary mechanisms by which isoflavones may prevent cancer in pre-menopausal women and certainly do not appear to have estrogenic-like effects in post-menopausal women.

Longer term supplementation than that carried out in this study, with thought given to the type of supplement (whole food or extracted supplement) and the subjects selected, may be required to fully elucidate this matter. This matter is of considerable importance in terms of the possible role of isoflavones in breast cancer prevention, where two early intervention studies focusing on the effects of phytoestrogens on the breast have both found evidence of a possible oestrogenic effect (McMichael-Phillips et al., 1998; Petrakis et al., 1996). It may be that the anti-cancer effects of isoflavones/soybeans are non-hormonal (Anderson, 1999).

5.4.4 IGF-1 and binding protein concentrations

Red clover isoflavone supplementation had no effect on IGF-1, IGFBP-1 or IGFBP-3 in post-menopausal women. In pre-menopausal women, isoflavone supplementation had no effect when multiple time points over the menstrual

cycle were considered, although there seemed to be a slight protective effect of isoflavone supplementation when the analysis was carried out simply before and after the supplementation period. This is likely to be due, however, to the large difference in IGF-1 levels at baseline and cannot be attributed to the isoflavone supplement. The effect of isoflavone supplementation on IGF status would therefore appear to be relatively weak. The clinical relevance of this small change may, in fact, be negligible. Our numbers were small, and the variance observed in IGF-1 and its binding proteins was high, so the area requires further study.

5.4.5 IGF and menstrual cycle

IGF-1 appears to be influenced by stages of the menstrual cycle. We observed a significantly higher concentration of IGF-1 and IGF-BP3 in pre-menopausal subjects on placebo in the luteal phase (days 21-23) compared to the menstrual phase (days 1-3). These changes over the menstrual cycle between the luteal and follicular phase are of a small magnitude (12.8% for placebo and 16.5% for supplement) and are in agreement with other studies (Caufriez et al., 1986; Juul et al., 1997; Lavigne et al., 2004). An early study in 5 premenopausal women (Caufriez et al., 1986) showed higher levels in the luteal (+4-+9) vs follicular (-3 - - 8) phases of the menstrual cycle and (Juul et al., 1997) also showed that, in ten healthy pre-menopausal women, there were significantly higher levels of IGF-1 in the luteal (0 - +10) vs follicular (-10 - 0) phase of the cycle ($P<0.01$). There were no cyclic changes in BP-3, while BP-1 had a pre-ovulation peak and was negatively correlated with SHBG levels. A more recent study in 37 pre-menopausal women (Lavigne et al., 2004) also found that IGF-1 increased by 7.6% in the luteal phase (days 21-23) and 3.3% in the preovulatory phase (days

12-15), compared with the follicular phase (days 5-7) (p=0.004), and saw no change in BP-3 levels. These small changes may explain why several other studies have not seen any significant cyclic variation in IGF-1, BP-1 or BP-3 (Blake et al., 1997; Guidoux et al., 1986; Helle et al., 1998; Thierry van Dessel et al., 1996; Wang et al., 1995).

A study in 9 pre-menopausal women (Helle et al., 1998) found IGF-1 and BP-3 levels to be highest in the follicular phase in 8 out of 9 women studied. However, the differences between the follicular and luteal phase were minimal with the major difference in plasma IGF-1 due to lower levels in the menstrual phase. As discussed previously (Duncan et al., 1999a), a feeding study in 14 pre-menopausal women (Wangen et al., 2000) found that IGF-1 was lower in the menstrual phase compared with the mid-follicular and mid-luteal phases (p=0.03 and p=0.05 respectively) whilst on the control ISP powder.

In this study, there were some consistent associations observed between IGF and binding proteins and hormones at certain stages of the menstrual cycle, but numbers were small, and the study not originally designed to examine this. In other studies, results for this have been conflicting. Positive correlations between oestradiol and IGF-1 have been seen (Helle et al., 1998; Ho et al., 1987; Massa et al., 1993) however others have found no correlations (Juul et al., 1997; Thierry van Dessel et al., 1996). A study by (Kelly et al., 1993) found that levels of serum oestrogen in post-menopausal women receiving exogenous oestrogens was actually negatively correlated with IGF-1 levels.

Certainly the positive correlations found in this study between BP-1 and SHBG levels throughout the menstrual cycle on both placebo and supplement reflects findings that both are regulated by insulin under various physiological conditions

(Holly, 1991) and has been seen in studies of post-menopausal breast cancer patients (Lonning et al., 1995).

These results therefore indicate that controlling closely for menstrual cycle phase may be necessary to observe IGF changes of small magnitude.

5.4.6 Isoflavones and IGF

To our knowledge this is the first published randomised controlled trial to examine the effects of isolated isoflavones on IGF-1 status in humans (Campbell et al., 2004). Since conducting this study between 1998-1999, other intervention studies have also published their findings on the effects of soy supplementation on IGF status **(Table 1.3.5.1)**, including one recent study that has also looked at isolated isoflavone supplementation in men.

Of the twelve studies published to date, half have seen a significant increase in serum IGF-1 concentrations while the others have seen no effect **(Table 1.3.5.1).** The discrepancy in results may be due to differences in endogenous estrogen levels between subjects studied i.e the study of males versus females, and pre or post-menopausal women or may be explained by the increased kilocalorie/ protein intake (Clemmons & Underwood, 1991) from the actual soy supplement used which may be masking the IGF lowering effects of isoflavones alone (Voskuil et al., 2005).

In animal studies, soy protein resulted in reduced IGF-1 in both normal and polycystic kidney disease affected male animals (Aukema & Housini, 2001), while Isoflavones reduced IGF-1 and inhibited growth of prostate cancer in mice (Zhou et al., 1999). It should be noted however that rodents convert most of their daidzein to equol (Setchell et al., 2002).

In our one week study using a soy, rye and linseed bar (see chapter 4) (Woodside et al., 2006), both IGF-1 and IGF-BP3 were significantly elevated after intervention.

Two studies were conducted using soy protein with a milk protein control. In men, serum IGF-1 was higher in those supplemented with soy protein than those consuming milk protein (Khalil et al., 2002). The same supplements given to post-menopausal women (Arjmandi et al., 2003) resulted in significant increases in IGF-1 on both soy protein ($p<0.0001$) and the milk protein control ($p=0.0045$). However like the men, the greatest increases in IGF-1 (97%) was seen in women on the Soy protein especially those who were not on HRT. These two studies would suggest that protein itself does increase IGF-1 but that soy protein had an effect on IGF-1 independent of its protein content. Calcium was not involved as both supplements had similar calcium content.

By contrast, in a three month study looking at markers of bone turnover (Wangen et al., 2000), in pre-menopausal women, IGF-1 and IGF-BP3 were increased by a low isoflavone diet, while in post-menopausal women, there were trends towards decreased IGF-1 and IGF-BP3 concentrations with increasing isoflavone concentration. The authors concluded that, although soy isoflavones do affect markers of bone turnover, the changes observed were of small magnitude and were not likely to be clinically relevant. In a study looking at soy protein supplementation with or with out 83 mg isoflavones (Adams et al., 2003), it was found that changes in serum IGF-1 and BP3 were similar in both groups and there appeared to be no effect of soy isoflavones on IGF-1 when compared to the protein supplement alone.

Similarly, a one year soy protein supplementation in post-menopausal women ($n=62$) (Arjmandi et al., 2005), found that both soy and dietary protein

supplements significantly increased serum IGF-1, however only those consuming soy protein had a significant increase in IGF-BP3.

In a 2 year soya intervention (~30mg isoflavones) in pre-menopausal women (Maskarinec et al., 2005b), there was also no significant change in serum IGF-1 or BP-3 throughout. They did see a positive correlation between total isoflavone excretion and IGF-1 ($p=0.004$). The author concluded that adding soya foods to the diet of pre-menopausal women does not appear to lower serum levels of IGF-1 and BP-3, if anything the greater protein intake from soy may lead to a small increase in IGF-1 serum levels.

There have recently been a few studies that have examined the effects of soy supplementation in combination with low fat/high fibre diets. A study in 17 prostate cancer patients (Spentzos et al., 2003) found that a low fat diet with the subsequent addition of a soy protein supplement (containing 114mg isoflavones), significantly increased serum IGF-1 levels from baseline ($p=0.02$), while a study by (Gann et al., 2005) found that in 153 pre-menopausal women randomised to the consumption of a low fat high fibre diet followed by soy protein with or without isoflavones vs the usual diet, there was no difference in the LFHF diet groups vs the usual diet. There were also no differences in IGF levels between those on soy with or without isoflavones. However, there was a small but significant decrease in BP-3 in both groups post soy protein supplementation regardless of isoflavone presence. In a recent study in post-menopausal women (Kaaks et al., 2003) involving five month dietary intervention with foods rich in dietary fibre/phytoestrogens (~45mg isoflavones) and a reduction in total fat and refined carbohydrate intake, there were also no significant changes in serum IGF-1 levels. However the intervention group did have increased levels of IGF-BP1, BP-2 and SHBG.

In all of the studies mentioned above it is difficult to disentangle the effects of isoflavones from soy protein (Voskuil et al., 2005) especially in those studies which have looked at soy supplementation as part of an overall healthy dietary intervention (Gann et al., 2005; Kaaks et al., 2003; Spentzos et al., 2003). Only our study (Campbell et al., 2004) and to our knowledge one subsequent study published recently (Vrieling et al., 2007), has looked at the effects of isoflavone supplementation on its own. The study by (Vrieling et al., 2007) also used a supplement derived from red clover (by the same manufacturer) and found that in the 37 men, with a family history of colorectal cancer or a personal history or adenomas, 84mg/d Isoflavone supplementation did not significantly affect serum IGF-1, BP-3 or BP-1 serum concentrations. However, they did find that the change in serum IGF-1 concentrations post supplement were negatively correlated to serum equol concentrations ($r=-0.49$, $p=0.002$). This requires further investigation in larger studies and as our numbers were small we cannot corroborate this finding in the 4 subjects who were classified as equol producers.

The results from our study and the intervention studies above are inconclusive but would point to there being little clinical effect of soy and isoflavones on serum levels of IGF-1. The results possibly show opposing effects of Isoflavones and soy protein, with IGF-1 decreased by isoflavones but increased by soy protein and possibly protein in general. Certainly feeding studies (Clemmons et al., 1985; Smith et al., 1995) have shown that a diet restricted of certain essential amino acids reduces IGF-1 levels possibly by decreasing production of GH which in turn reduces hepatic production of IGF-1 (Ketelslegers et al., 1995) and increases BP-1 (Jousse et al., 1998). The status of endogenous oestrogen concentrations may also contribute to the differences

seen with IGF-1 association between men and women, pre and post-menopausal, HRT vs none, etc.

However further larger and long-term intervention studies using isolated isoflavone supplementation should determine whether the isoflavone content of soy is beneficial to IGF status.

Since this study was conducted there have also been a number of cross-sectional studies published that have looked at links between dietary intake and IGF-1 concentrations **(Table 1.3.4.1)**. Some of the studies have been reviewed (Voskuil et al., 2005) and a few of them have also examined associations with intake of Soy/phytoestrogens (Nagata et al., 2003; Probst-Hensch et al., 2003; Vrieling et al., 2004). No correlations were seen between soy product intake and IGF-1 or BP-3 levels in 261 Japanese pre-menopausal women (Nagata et al., 2003) or the 386 pre and post-menopausal Dutch women (Vrieling et al., 2004) assessed using food frequency questionaires. However, there was a positive association found between soy intake and IGF-1 in 321 Chinese men studied (Probst-Hensch et al., 2003) and a positive association with plant lignans and BP1 concentrations in 162 post-menopausal Dutch women (Vrieling et al., 2004). It would appear then that the findings from the cross-sectional studies are in agreement with results from our study and the other intervention studies, i.e. there appears to be very little effect of soy/isoflavones on IGF-1 concentrations and that, rather than reducing levels of IGF-1, consumption of soy may in fact be increasing levels particularly in men and post-menopausal women who are not on HRT (Arjmandi et al., 2003; Khalil et al., 2002).

Other nutritional factors that have been associated with IGF-1 levels are energy and protein intake, with malnutrition and fasting states resulting in significant reductions of serum IGF-1 levels (Thissen et al., 1994). The physiological

mechanisms are thought to be linked to low levels of insulin, resulting in consistently high levels of GH, which leads to hepatic resistance and binding of GH, with decreased production of hepatic IGF-1 (Thissen et al., 1994). However, these correlations only appear to apply with extreme dietary restictions as only a few cross-sectional studies, done in well-nourished individuals, have shown positive associations with energy intake (Heald et al., 2003) where subjects with BMI<25 kg/m^2 are analysed separately (Giovannucci et al., 2003; Holmes et al., 2002). We did not see a positive association between IGF-1 and total energy intake but we did find a number of positive correlations with anthropometric data **(Table 5.A.18)** including correlations between IGF-1 and waist, weight and hip measurements. We also did not detect any correlations with protein intake and IGF-1 in pre-menopausal women. Ten of the seventeen crossectional studies done to date **(Table 1.3.4.1)** did not see any significant correlations between IGF-1 and protein intake. Seven saw positive associations with protein intake (Allen et al., 2002; Devine et al., 1998; Holmes et al., 2002), (Giovannucci et al., 2003; Heald et al., 2003; Larsson et al., 2005; Norat et al., 2007) and only one study (Maskarinec et al., 2005a) saw a suggestive negative corrleation with IGF-1 and protein in the form of fish intake.

This study showed some associations between serum levels of triglycerides, total cholesterol and IGF-1 concentrations in pre-menopausal women, which is consistent with findings by (Probst-Hensch et al., 2003) which also found strong positive associations between IGF-1 concentrations and serum levels of cholesterol, LDL and triglycerides. Other cross-sectional studies have examined fat intake through dietary questionnaires and have found significant positive correlations with IGF-1 concentrations (Baibas et al., 2003; Gunnell et al., 2003;

Heald et al., 2003; Kaklamani et al., 1999), with one study shoing a negative association between intake of fat from meat and IGF-1 (DeLellis et al., 2004). Interestingly we found that, in pre-menopausal women, BP-1 levels were consistently positively associated with HDL levels and as there have been no other studies to examine this link it would be worth further investigation.

Studies linking the IGF system with the development of cardiovascular disease (Bayes-Genis et al., 2000), have been examined fairly recently, finding associations between insulin like growth factors and its binding protiens with serum lipid levels, (Fischer et al., 2004; Harrela et al., 2002; Janssen et al., 1998; Juul et al., 2002; Laughlin et al., 2004).

The positive associations seen in our pre-menopausal women between BP-1 and HDL, agree with findings by (Janssen et al., 1998) and (Laughlin et al., 2004), while the positive associations for IGF-1 with triglycerides and cholesterol (although not consistent at all timepoints) appear to agree with findings by (Laughlin et al., 2004) but not (Harrela et al., 2002; Juul et al., 2002). The links are not entirely clear, however, with (Juul et al., 2002) showing that low IGF-1 levels puts patients at significantly higher risk of developing ishaemic heart disease (IHD), while recent findings by (Fischer et al., 2004) suggest a postive association with IGF-1, BP-3 levels and CVD. A review by (Juul, 2003) suggesting that the controversy around clinical studies of IGF-1 being either cardioprotective or a risk fator for IHD may reflect that IGF-1 was studied at different time points in relation to the ishaemic heart event.

Although none of the cross-sectional studies done to date have looked at associations with serum levels of antioxidants, the negative correlations we found between IGF-1 and its binding proteins and serum levels of carotenoids are similar to findings by the recent large EPIC crossectional study examining

diets of women in Europe (Norat et al., 2007) which saw negative correlations with dietary intake of β-carotene, while an earlier study of 112 Greek men (Mucci et al., 2001) showed that greater consumption of cooked tomatoes (the largest source of lycopene) was associated with lower concentrations of IGF-1 ($p=0.041$). However, positive correlations have also been observed in cross-sectional studies (Holmes et al., 2002; Maskarinec et al., 2005a).

There also appears to be a strong suggestion that milk and/or dairy consumption is associated with higher levels of IGF-1, with many of the crossectional studies showing positive correlations with IGF-1 concentrations and milk/dairy foods (Gunnell et al., 2003; Ma et al., 2001; Norat et al., 2007) and/or calcium and vitamin D intake (Giovannucci et al., 2003; Gunnell et al., 2003; Holmes et al., 2002; Nagata et al., 2003; Norat et al., 2007; Probst-Hensch et al., 2003). While consumption of ¾ pint of soya milk daily in vegan women significantly increased IGF-1 concentrations (Allen et al., 2002), these increases in IGF-1 could be attributable to increased levels of protein (including essential amino acids and possibly ingestion of bovine IGF-1 contained in milk (Giovannucci, 2003). Other nutrients that have been shown to be positively associated with IGF-1 include intake of vitamin B6 and B12 (Norat et al., 2007). (Maskarinec et al., 2005a), found that women in the top quartiles of dietary fiber intake had 5.7% higher IGF-1 levels than women in the bottom quartiles ($p=0.004$).

We did not see any associations with IGF levels and alcohol consumption unlike other studies (Probst-Hensch et al., 2003; Vrieling et al., 2004), nor did we find any associatons between Zinc intake and IGF-1 (Devine et al., 1998; Giovannucci et al., 2003; Holmes et al., 2002; Larsson et al., 2005).

Due to the small numbers in our study it is difficult to draw conclusions from the correlations found between serum levels of lipids and antioxidants, dietary intake and IGF-1, BP3 and BP1 levels. Further larger studies and intervention studies should be done to determine if there is a causal effect and what the relationships are with these dietary components, including analysis of serum concentrations of these nutrients which may give a more accurate assessment of dietary intake.

5.4.7 Isoflavones and lipids

We have shown an effect of isoflavone supplementation within one month on HDL-cholesterol in post-menopausal subjects. Several studies have provided evidence that phytoestrogens in general can modulate plasma lipid and lipoprotein concentrations, but the data are not entirely consistent (Anderson et al., 1995). A recent overview concluded that soy could improve blood lipid parameters in both normocholesterolaemic and hypercholesterolaemic subjects, although the use of soy alone may not allow patients with hyperlipidaemia to achieve target lipid parameters (Costa & Summa, 2000). Soy, with which most of the studies have been carried out, has a variety of properties unrelated to isoflavone content that may contribute to its lipid-modulating properties (Potter, 1995). Our post-menopausal subjects had normal lipid levels, but displayed an increase in HDL-cholesterol during the isoflavone supplementation phase. This may be pertinent in light of the fact that, a short-term study using the same purified red clover isoflavone pills found no effect on plasma lipids and lipoprotein concentrations (Nestel et al., 1999) and another found only small changes in levels of triglycerides (Schult et al., 2004). However other longer term studies using this supplement have demonstrated an effect of isoflavones

on HDL_3 (Samman et al., 1999) in pre-menopausal women and total HDL (Clifton-Bligh et al., 2001; Knight et al., 1999) in pre-menopausal and post-menopausal women. A recent study in post-menopausal women with a different isoflavone supplement not derived from red clover (containing 47mg as aglycone form) found that HDL cholesterol significantly increased from baseline in the combination of walking and supplement but not on supplement alone (Wu et al., 2006). The apparent difference in these findings compared to those obtained with soy-derived isoflavones (Dewell et al., 2002; Gardner et al., 2001; Somekawa et al., 2001) could be accounted for by the high levels of biochanin and fomonomentin in red clover isoflavone extracts.

5.4.8 Isoflavones and antioxidants

Isoflavones can act as antioxidants (Bingham et al., 1998), both *in vitro* and test systems. (Kapiotis et al., 1997) observed that LDL oxidation products, assayed as thiobarbituric acid-reactive substances, were strongly inhibited by genistein, somewhat inhibited by daidzein, but not affected by genistin (the glycosylated form of genistein) or control. Furthermore, both genistein and daidzein were found to protect against cytotoxic effects of oxidised LDL as assessed by cellular morphologic features and lactate dehydrogenase release by cultured endothelial cells. Genistein has also been shown to inhibit hydrogen peroxide production and increase the activity of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase (Lissin & Cooke, 2000). A protective/antioxidant effect has been shown by phytoestrogen supplementation on the susceptibility of LDL to oxidation in another study (Tikkanen et al., 1998). Six healthy volunteers received 19 mg isoflavones/day for 2 weeks. Lag phases of LDL oxidation curves were reduced

post-supplement compared with baseline values. Another study, however, failed to show an inhibition of lipid peroxidation after 8 weeks of isoflavonoid supplementation in subjects with high-normal blood pressure (Hodgson et al., 1999), measuring urinary F-2-isoprostanes. Another study in 24 subjects showed that consumption of soy containing burgers containing 21mg Daidzein and 35mg genistein isoflavones, reduced lipid peroxidation in plasma in the form of plasma F(2)isoprostane and increased the resistance of LDL to oxidation (Wiseman et al., 2000). We found little effect of isoflavone supplementation on markers of antioxidant status. Our subjects were healthy and had normal antioxidant levels; it may be that isoflavones will exert marked antioxidant effects *in vivo* only in those with an already compromised antioxidant status.

5.4.9 Diet

Background diet did not seem to affect the findings of this study, with the magnitude of any differences observed being unlikely to affect our conclusions. Overall analysis of dietary intake showed that mean intakes were comparable to national averages in the UK (Swan, 2004). The percentage of calories from protein, carbohydrate, fat and alcohol were very similar between FFQs and FDs. In terms of biomarkers, overall serum levels of carotenoids appeared to correlate strongly with both FFQ and FDs. Serum lipid levels of LDL, cholesterol and MDA showed some correlation with FFQ estimated intakes of fats but not at all with intakes estimated from FDs. The FFQ estimated intake of calories, cholesterol, % fat and fibre also showed some interesting correlations with anthropometric data.

Thus, it would appear that both methods of dietary assessment showed a reasonable level of agreement. However the FFQ appeared to perform better when compared with biomarkers and anthropometric data. Therefore the FFQ may well give a better indication of long-term dietary habits.

5.5 Conclusion

Red clover-derived isoflavone supplementation over one month has no effect on IGF status in post-menopusal women, and at most has a small effect on IGF-1 in pre-menopausal women. Red clover isoflavones seem to increase HDL-cholesterol in post-menopausal women, but have little or no effect on other lipids or antioxidant status. It must be remembered, in the interpretation of these data, that the study was small and had low power to detect a difference between placebo and supplement, particulary when multiple comparisons were being carried out. Further work in larger numbers will determine whether these dietary compounds may play a role in endocrine-responsive cancer and Cardiovascular disease prevention.

CHAPTER SIX – THE EFFECT OF ISOFLAVONES ON DNA SYNTHESIS IN MCF-7 AND MDA-MB-231 HUMAN BREAST CANCER CELLS

6.1 Introduction

In vitro, isoflavones inhibit the growth of both oestrogen dependent and independent breast cancer cells by a number of different mechanisms (Dampier et al., 2001; Magee & Rowland, 2004; Peterson & Barnes, 1991; Sarkar & Li, 2003).

Isoflavones bind to mammalian oestrogen receptors with between 1 x 10^{-2} and 1 x 10^{-4} the activity of 17β-oestradiol and are conventionally considered weak oestrogenic compounds (Markiewicz et al., 1993). However the discovery of the oestrogen receptor β subtype (ERβ) (Kuiper et al., 1996; Kuiper & Gustafsson, 1997) has added a new dimension to the understanding of oestrogen and anti-oestrogenic actions of these compounds. The relative binding affinities and transcription activities of isoflavones compared to oestrogen, in both α and β subtypes have been investigated (Kuiper et al., 1997; Kuiper et al., 1998). Isoflavones, and in particular genistein and equol, have 30-100 fold higher binding affinities and transcription activities for ERβ compared with ERα (An et al., 2001; Barkhem et al., 1998; Kostelac et al., 2003) (McCarty, 2006).

Normal and malignant breast tissue express both receptors (Kuiper et al., 1997). Studies suggest that ERβ is the principle oestrogen receptor in normal breast tissue with decreasing expression in poorly differentiated cancerous and metastatic tissue (Palmieri et al., 2002), with others finding that ERβ is upregulated in breast tumours that develop antioestrogen resistance (Speirs &

Kerin, 2000). Although the precise role of ERβ activation is unclear (Park et al., 2003) there is evidence that it opposes the proliferative effects of ERα *in vitro* (Caetano et al., 2006; Paruthiyil et al., 2004; Strom et al., 2004).

Expression of the oestrogen receptors in breast cancer cell lines, shows that the ER positive MCF-7 cells strongly express ERα, while both MCF-7 and the ER negative MDA-MB-231 cells express low levels of ERβ (Dampier et al., 2001; Fuqua et al., 1999; Tong et al., 2002; Vladusic et al., 2000).

Genistein and daidzein bind to ERα with 4% and 0.1% affinitiy of oestradiol respectively (Kuiper et al., 1998). Equol has similar binding affinity to genistein but a much stronger ability to induce transcription (Morito et al., 2001). In ERα positive MCF-7 cells, isoflavone concentrations ≤1μM increase cell proliferation, with genistein shown to be a strong oestrogenic agonist for both ER isoforms (Maggiolini et al., 2001). However, higher concentrations (≥10μM) have been shown to inhibit hormone dependent growth by oestrogen antagonistic activity, and these biphasic effects on cell growth are now well documented (Fioravanti et al., 1998; Hsieh et al., 1998; Hsu et al., 1999; Le Bail et al., 1998; Power & Thompson, 2003; Wang & Kurzer, 1997; Wang et al., 1996; Zava & Duwe, 1997). Research by (Hsieh et al., 1998) found that concentrations as low as 10nM of genistein could have proliferative effects in MCF-7 cells, with 100nM achieving proliferative effects similar to 1nM of oestradiol. Others have also found that genistein and equol induced oestrogen regulated pS2 expression at concentrations of 1nM to 1μM, with their growth stimulation linked to the binding affinity for ERα (Zava & Duwe, 1997). They also found that genistein's ability to override the growth inhibitory effects of tamoxifen helped demonstrate that the inhibitory mechanisms of isoflavones may not be the same as classical oestrogen agonists like tamoxifen. (Le Bail et al., 1998) found that, in the

presence of 0.1nM oestradiol, the proliferative effects of isoflavones at lower concentrations were no longer present while their inhibitory effects at higher concentrations (~50μM) were unaffected by oestradiol.

Oestradiol and IGF-1 appear to work together in the proliferation of breast cancer cells with increasing suppport for cross talking between the ER and IGF-1R signalling pathway (Kato et al., 2000; Laban et al., 2003). Oestradiol has been showen to stimulate expression of IGF-1, the IGF-1R, IRS-1 and down regulate expression of the IGF binding proteins (Ruan et al., 1995; Stewart et al., 1990), (Huynh et al., 1996b; Lee et al., 1999). The cytotoxic effects of tamoxifen are partially mediated through IGF-1 receptor (IGF-1R) signalling (Guvakova & Surmacz, 1997), while genistein has recently been shown to enhance the IGF-1 signalling pathway in proliferation of MCF-7 cells at low concentrations (Chen & Wong, 2004).

In ERα negative cells, isoflavones do not appear to cause biphasic effects on cell proliferation. A study comparing the effects of genistein in different breast cancer cell types, found that growth promotion at low concentrations of 1-10μM were only apparent in MCF-7 cells, and that different mechanisms were found to promote growth depending on breast cell type (Dampier et al., 2001). Whether these antiproliferative effects are mediated through binding to ERβ has yet to be fully examined. However research by (Caetano et al., 2006) has recently found that in MDA-231 cells, genistein increases expression of all genes involved in cell cycle control, apoptosis and DNA repair including BRAC1, p53, p21, Rad51, BARD, Bax GADD45 and RF1, suggesting it may decrease the inhibition exerted by oestrogens on the transcription of these genes by antagonising ERβ.

A recent study using cDNA miocroarray technology has shown that 50μM Genistein can also upregulate genes such as HSP, involved in cell salvage response, and down regulate SRF transcription factor, involved in cell growth /differentiation through oestrogen receptor independent pathways (Chen et al., 2003). Other oestrogen independent mechanisms have been reviewed (Middleton et al., 2000; Sarkar & Li, 2003), and include: inhibition of AKt signalling and activation of nuclear fator KB DNA binding activity needed for cell survival, inhibition of antiproliferative enzymes such as topoisomerase I and II and 5α reductase, inhibition of protein tyrosine kinase mediated signalling pathway, inhibition of angiogenesis and metastasis, sitmulation of immunity and antioxidant activity (Akiyama et al., 1987; Akiyama & Ogawara, 1991; Chen et al., 2003; Kim et al., 1998; Ruiz-Larrea et al., 1997; Shao et al., 1998a; Shao et al., 1998b; Sierens et al., 2001; Su et al., 2005; Zhang et al., 1999).

However, caution is warranted in the interpretation of some of these effects, as they have been observed at higher concentrations than may be found *in vivo*. Reports on plasma concentrations of isoflavones do vary, with levels reported to reach no higher than 18.5μM (Barnes & Peterson, 1995). Soy rich Japanese diets can result in total concentrations above 5μM (Morton et al., 1994) with mean concentrations of genistein in Japanese men (493nM) compared with men from UK (33nM). It was found that 58% of Japanese men and 38% of women had serum equol concentrations above 20nM compared with none and 2.2% of comparable samples in the UK population (Morton et al., 2002). Total levels of isoflavones following moderate consumption of soy are reported to be ~2μM (Franke et al., 1998) with studies following consumption of genistein rich foods, finding that plasma concentrations are generally <1μM of genistein (Barnes et al., 1996), (Adlercreutz et al., 1995a; Gooderham et al., 1996;

Wiseman et al., 2004; Xu et al., 1994). Recent bioavailbilty studies looking at the American caucasian female population have found peak plasma genistein concentrations of 0.5-2.2 μM after 4-8 hours of ingestion (Setchell et al., 2003), with the bioavailability from aglycone forms higher than glycosides for daidzein with no difference seen for genistein (Zubik & Meydani, 2003). Studies suggest that as little as ~0.5-2% of genistein in serum is actually available as the unbound unconjugated form of genistein (Barnes, 1995; Nagel et al., 1998) which may be equivalent to ~10nM genistein concentrations *in vitro* (McCarty, 2006).

However, cellular concentrations may in fact be higher than this, with oestrogen concentrations found to be up to 40 fold higher in breast ductal fluid than in serum (Ernster et al., 1987). A study by (Hargreaves et al., 1999) also found significantly higher isoflavone levels in nipple aspirate fluid than in serum, in both control and soy supplemented pre-memopausal women, suggesting accumulation of phytoestrogens within breast ducts. Similar concentrated effects have also been seen in prostatic fluid (Morton et al., 1997). However, (Pumford et al., 2002) found that breast tissue levels of phytoestrogens are comparable to serum levels, and increase with isoflavone supplementation.

In vitro studies in breast cancer cells can improve our understanding of the possible therapeutic benefits that isoflavone compounds may have. Thus, the present study was designed to investigate the effects of isoflavones on DNA proliferation in the oestrogen dependent, non-invasive MCF-7 breast carcinoma cell line and the oestrogen-independent, invasive MDA-MB-231 breast carcinoma cell line. Interactions between the isoflavones, antiestrogen Tamoxifen and IGF-1 were also examined to investigate possible mechanisms

of action. To examine the mechanisms by which isoflavones inhibit cell growth, concentrations of 10μM (~IC50) of isoflavones were combined with:

- 1nM Oestradiol to imitate levels seen in plasma for premenopausal women (Pike et al., 1993),
- 10nM IGF-1 which gave near maximum promotion of DNA synthesis in both cell lines
- 1μM tamoxifen to imitate levels in women receiving tamoxifen for prevention/treatment of breast cancer (Lien et al., 1989) and can inhibit ER and IGF-1 mediated proliferation of MCF-7 cells (Jordan, 1994) (Wosikowski et al., 1993).

6.2 Materials and methods

6.2.1 Cell Culture

MCF-7 and MDA MB -231 cells (kindly donated by Ludwig cancer research group) were maintained in Phenol Red IMEM (GIBCO) supplemented with 5% FCS (GIBCO), 1% L-Glutamine (sigma) and 1% Fungizone (GIBCO) in 5% CO_2 at 37°C. Conditions involved growing cells in phenol red free IMEM supplemented with 5%FCS for 72 hours prior to plating. Cells were then seeded in 96 well plates (NUNC) at 1x 10^4 cells per well in 5% DCC-FCS (Dextran charcoal coated foetal calf serum) phenol red free IMEM and incubated for 24 hours. Medium was changed to serum free IMEM for another 24 hours before the compounds were added. Stock solutions of tamoxifen, genistein (4,5,7-trihydroxyisoflavone), daidzein (4,7-dihydroxyisoflavone), equol and 17β-oestradiol (Sigma) in ethanol were used to produce the required concentrations of compounds in culture medium. Control conditions also contained the appropriate amount of ethanol (0.1%).

6.2.2 BrdU assay

The principle of the assay is to determine cell proliferation by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis in proliferating cells. This assay indicates growth rate as opposed to cumulative cell growth. Conventional methods use thymidine, however this technique is based on the incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU). The BrdU assay was performed using a colorimetric ELISA kit (Amersham-Pharmacia-Biotech, 1998). Results were from five replicate wells with experiments performed two to three times.

See Chapter two for further detail on Methods.

6.3 Results

Because we have not directly compared DNA synthesis with other assays, such as cell number or protein content, we cannot assume that these results are directly comparable. However, previous studies have compared assays and found DNA synthesis to parallel other assays and directly correlate with the [^{3}H]thymidine incorporation assay (Amersham-Pharmacia-Biotech, 1998). The work presented here does appear to correlate quite closely with results from studies that have used thymidine to analyse DNA synthesis in these cell types, and could therefore be considered as a useful and safer alternative.

6.3.1 Effects of E_2 and IGF-1 on DNA synthesis

DNA synthesis was significantly stimulated with E_2 after 24 hour incubations in MCF-7 cells **(Table 6.3.1.1** and **Fig 6.3.1.1)**. Induction was 16%, 173%, 187% and 159% of the 0% control at 0.01nM, 0.1nM, 1nM and 10nM, respectively. ($P<0.01$). In MDA-MB-231 cells E_2 did not stimulate DNA synthesis but appeared to inhibit cells to between 87 and 89% of the control at higher concentrations ($P<0.05$) **(Table 6.3.1.2** and **Fig 6.3.1.2)**.

IGF-1 significantly increased DNA synthesis in MCF-7 cells to 123%, 149%, 198% and 214% of the control at 0.1nM, 1nM, 10nM and 100nM, respectively ($P<0.01$) **(Table 6.3.1.1** and **Fig 6.3.1.1)**. In MDA-231 cells IGF-1 did not stimulate DNA synthesis but inhibited to 92 and 89% of the control at higher concentrations ($P<0.05$) **(Table 6.3.1.2** and **Fig 6.3.1.2)**.

6.3.2 Effects of Isoflavones and Tamoxifen on DNA synthesis

The isoflavones genistein, daidzein and equol all stimulated DNA sunthesis to a maximum of 207%, 198% and 209% at 1μM respectively. Both genistein and

equol caused significant stimulation between concentration of 0.01μM-10μM, ($P<0.01$), while daidzein significantly increased stimulation between 0.1μM-100μM, ($P<0.01$). Biphasic effects, meant that at higher concentrations, genistein and equol were able to inhibit DNA synthesis, with IC_{50} values of 93 and 55μM respectively. Tamoxifen did not have biphasic effects and inhibited DNA synthesis from 0.1μM ($P<0.05$) through to 100μM ($P<0.01$) with an IC_{50} value of 1.17μM **(Table 6.3.1.1** and **Fig 6.3.2.1)**.

In MDA-MB-231 cells the isoflavones did not stimulate DNA synthesis at any concentration but significantly inhibited induction as concentrations increased. Genistein inhibited DNA synthesis from concentrations of 0.1μM ($P<0.01$) upwards with an IC_{50} value of 14μM. Daidzein and equol inhibited DNA synthesis at concentrations greater than 1μM ($P<0.01$) with an IC_{50} value of 15μM for equol. Tamoxifen inhibited synthesis form concentrations of 0.1μM ($P<0.01$) onwards with an IC_{50} value of 1.35μM **(Table 6.3.1.2** and **Fig 6.3.2.2)**.

Table 6.3.1.1: Dose response of MCF-7 cells incubated with Isoflavones, Tamoxifen, IGF-1 and oestradiol for 24 hours.

	0.01uM	**0.1uM**	**1.0uM**	**10uM**	**100uM**	**IC_{50}**
Daidzein	96.4(15.6)	153.5(34.6)*	198.0(50.5)*	161.2(60.2)*	133.8(41.2)*	>100μM
Genistein	163.8(29.7)*	197.7(25.3)*	207.1(43)*	156.3(39.2)*	41.0(27.4)*	93μM
Equol	122.4(14.6)*	193.8(43.2)*	209.1(54.2)*	141.5(45.4)*	14.7(1.9)*	54.8μM
Tamoxifen	92.5(9.4)	91.5(8.0)*	59.7(11.6)*	7.45(0.7)*	9.11(0.8)*	1.17μM
	1pM	**0.01nM**	**0.1nM**	**1nM**	**10nM**	**100nM**
IGF-1		102.9(14.4)	123.3(26.0)*	149.2(18.0)*	197.6(45.2)*	214.3(46.5)*
Oestradiol	97.4(17.7)	160.9(22.9)*	172.9(25.9)*	187.4(45.8)*	159.3(39.2)*	

Levels given as percentage of the control. Mean (SD) *P<0.01 Mann Whitney U test.

Table 6.3.1.2: Dose response of MDA-231 cells incubated with Isoflavones, Tamoxifen, IGF-1 and oestradiol for 24 hours.

	0.01μM	**0.1μM**	**1.0μM**	**10μM**	**100μM**	**IC_{50}**
Daidzein	100.4(9.3)	104.5(11.2)	98.3(12.1)	80.4(8.1)*	54.0(7.9)*	113μM
Genistein	96.4(10.3)	89.5(5.7)*	85.6(7.8)*	59.7(10.5)*	19.5(5.4)*	14.1μM
Equol	94.0(7.0)	100.8(7.7)	97.4(14.9)	65.2(11.0)*	9.0(1.7)*	14.55μM
Tamoxifen	96.1(5.6)	86.9(6.8)*	74.5(12.4)*	4.7(0.3)*	5.1(0.5)*	1.35μM
	1pM	**0.01nM**	**0.1nM**	**1nM**	**10nM**	**100nM**
IGF-1		101.3(6.2)	106.7(12.3)	101.1(10.5)	91.7(9.2)*	89.3(5.8)*
Oestradiol	97.4(13.0)	91.5(13.5)*	88.7(8.4)*	92.6(9.4)*	86.7(15.0)*	

Levels given as percentage of the control. Mean (SD) * P<0.01 Mann Whitney U test.

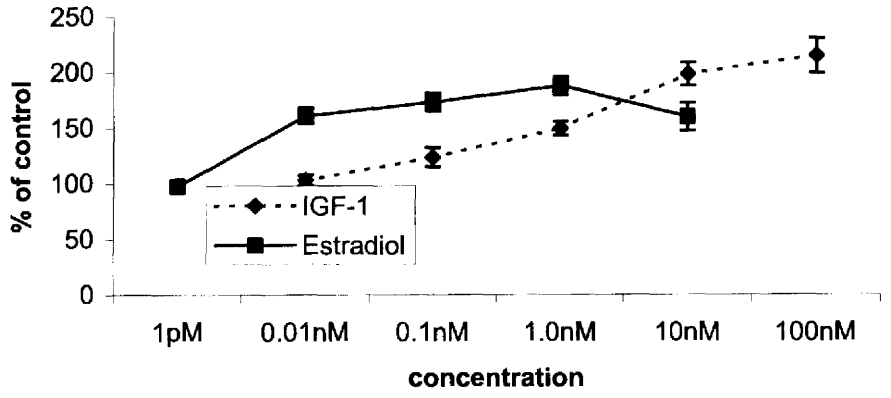


Figure 6.3.1.1: Dose response of MCF-7 cells incubated with IGF-1 and oestradiol for 24 hours before labelling with BrDU. Mean (SE)

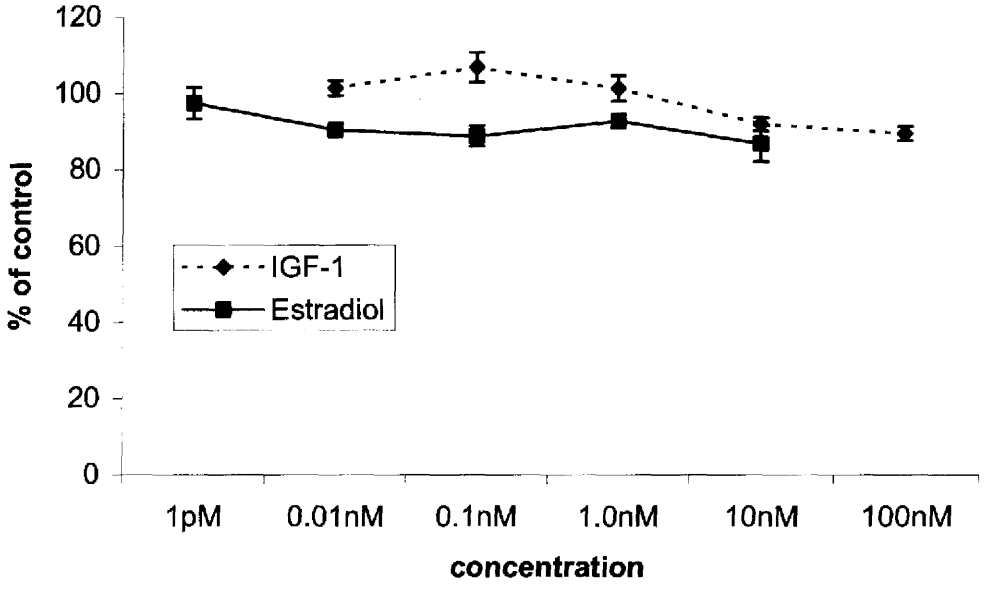


Figure 6.3.1.2: Dose response of MDA-MB-231 cells incubated with IGF-1 and oestradiol for 24 hours before labelling with BrDU. Mean (SE)

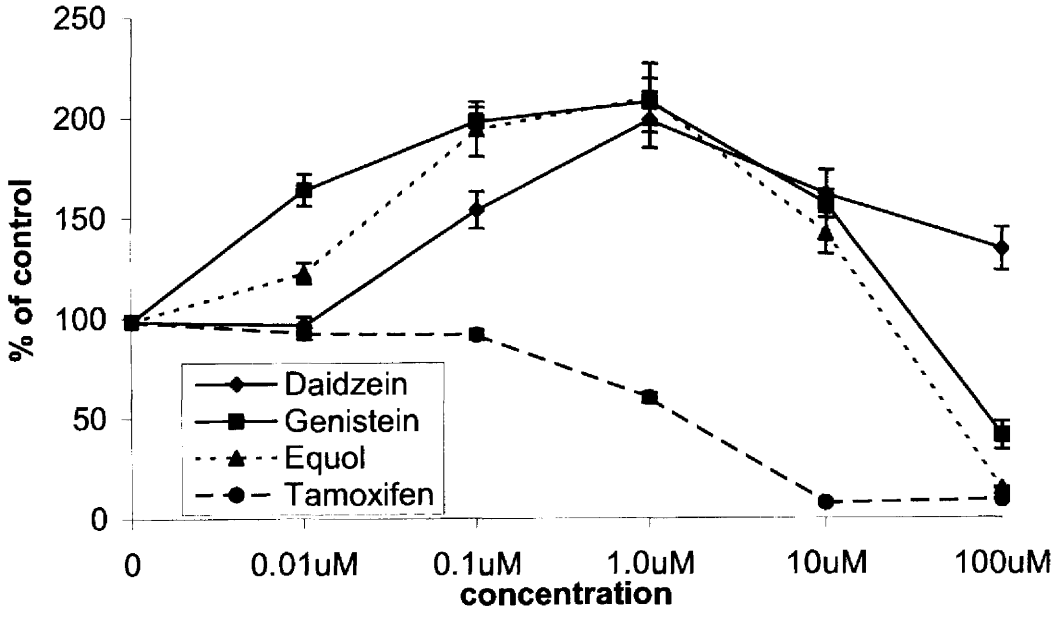


Figure 6.3.2.1: Dose response of MCF-7 cells incubated with isoflavones and Tamoxifen for 24 hours before labelling with BrDU. Mean (SE)

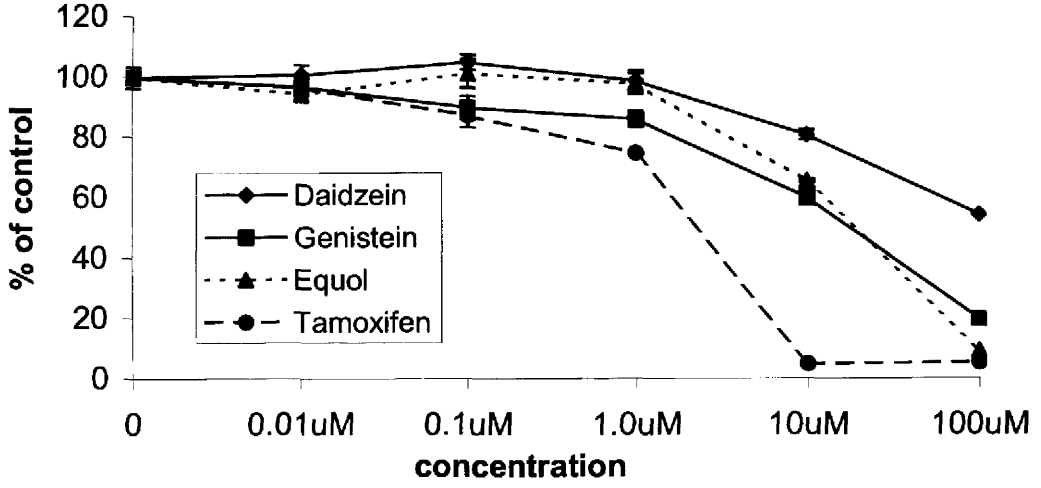


Figure 6.3.2.2: Dose response of MDA-MB-231 cells incubated with isoflavones and Tamoxifen for 24 hours before labelling with BrDU. Mean (SE)

6.3.3 Interactions between isoflavones and Tamoxifen with Oestradiol

In MCF-7 cells maximal stimulation of DNA synthesis with 1nM E_2 alone was 187% of the control. When incubated in combination with 10μM genistein, daidzein and equol, synthesis induced by 1nM E_2 was decreased by 51%, 41% and 43% respectively ($P<0.01$).

When incubated in combination with 1μM tamoxifen, the decrease of E_2 induced synthesis was 28% ($P<0.01$) **(Table 6.3.3.1)**.

In MDA-231 cells the inhibition caused by isoflavones alone was counteratcted by the addition of 1nM Estradiol such that the inhibition of DNA synthesis was reduced by 10%, 7% and 11% for genistein, daidzein and equol respectively ($p<0.01$).

This was not the case with tamoxifen as there was very little difference seen between inhibition with or without E_2 **(Table 6.3.3.2)**.

6.3.4 Interactions between Isoflavones with Tamoxifen

In MCF-7 cells, 1μM tamoxifen inhibited DNA synthesis by 60% ($P<0.01$). However, when added to 10μM genistein and daidzein, tamoxifen increased DNA synthesis by a further 22% and 15% ($P<0.05$). This increase was not seen when tamoxifen was added to equol **(Table 6.3.3.1)**.

In MDA-231 cells, 1μM tamoxifen inhibited DNA synthesis to 74.5% of the control. Unlike the MCF-7 cells, the addition of genistein and equol reduced this inhibition further by 11% ($P<0.01$) and 22% ($P<0.01$) **(Table 6.3.3.2)**.

6.3.5 Interactions between Isoflavones and Tamoxifen with IGF-1

Stimulation of DNA synthesis with 10nM IGF-1 was 198% of the control ($P<0.01$). When incubated with isoflavones, induction was decreased. With

equol, this was a significant reduction of 46.2% ($P<0.01$). Tamoxifen also reduced IGF-1 induced synthesis but this was not significant. No significant increase in DNA synthesis was seen with the addition of E_2 to IGF-1 **(Table 6.3.3.1)**.

In MDA-231 cells the addition of 10nM IGF-1 reduced the ability of genistein, daidzein and equol to inhibit DNA synthesis by 11%, 20% and 17%, respectively ($P<0.05$). However, this was not the case with tamoxifen as there was no difference seen in the amount of DNA inhibition between tamoxifen on its own or combined with IGF-1. There was a small yet significant increase in DNA synthesis with the addition of E_2 to IGF-1 ($P<0.01$) **(Table 6.3.3.2)**.

Table 6.3.3.1 Response of MCF-7 cells incubated for 24 hours with different combinations of Isoflavones, oestradiol, Tamoxifen and IGF-1.

		Oestradiol(1nM)	**Tamoxifen**(1μM)	**IGF-1**(10nM)
Genistein(10μM)	156.3(39.2)	136.9(7.9)	178.6(17.2)	168.7(9.2)
Daidzein(10μM)	161.2(60.2)	146.5(12.9)	176.2(17.6)	181.3(12.4)
Equol(10μM)	141.5(45.4)	144.6(13.4)	143.2(15.2)	151.4(22.3)
Tamoxifen(1μM)	59.7(11.6)	159.0(49.8)	-	169.6(41.0)
Oestradiol(1nM)	187.4(45.8)	-	159.0(49.8)	190.2(9.6)
IGF-1(10nM)	197.6(45.2)	190.2(9.6)	169.6(41.0)	-

Levels given as a percentage of the control. Mean (SD)

Table 6.3.3.2: Response of MDA231 cells incubated for 48 hours with different combinations of Isoflavones, oestradiol, Tamoxifen and IGF-1.

		Oestradiol(1nM)	**Tamoxifen**(1μM)	**IGF-1**(10nM)
Genistein(10μM)	59.7(8.1)	69.9(4.3)	63.4(6.0)	70.2(12.8)
Daidzein(10μM)	80.4(10.5)	87.0(6.8)	91.4(6.6)	100.2(18.6)
Equol(10μM)	65.2(11.0)	76.4(4.0)	52.9(5.1)	81.9(14.2)
Tamoxifen(1μM)	74.5(12.4)	68.2(12.9)	-	71.1(9.1)
Oestradiol(1nM)	92.6(9.4)	-	68.2(12.9)	102.1(11.2)
IGF-1(10nM)	91.7(9.2)	102.1(11.2)	71.1(9.1)	-

Levels given as a percentage of the control. Mean (SD)

6.4 Discussion

6.4.1 Isoflavones

DNA synthesis was significantly increased by E_2 in MCF-7 (ERα+/ERβ+) cells but not MDA-MB-231 (ERα-/ERβ+) cells. These results confirm the role of ERα in facilitating the dose dependent proliferation effects of E_2 in MCF-7 cells (Dampier et al., 2001). The isoflavones genistein, daidzein and its metabolite equol also significantly stimulated DNA synthesis in a dose dependant manner in MCF-7 cells. Maximal stimulation for all three isoflavones tested was reached at 1.0 uM, with an increase of 207%, 198% and 209% for genistein, daidzein and equol respectively. However, genistein and equol concentrations >10μM inhibited DNA synthesis with IC50 values of 93μM and 54.8μM respectively. This biphasic response of MCF-7 cells to increasing concentrations of isoflavones has also been reported in other studies looking at DNA synthesis. Incubation for 24 hours with 1uM genistein and 10uM daidzein in the presence of 5% DCC-fCS, maximized DNA synthesis to 215% and 210% of the control while, inhibition occurred between 50-100μM with an IC50 value for genistein of 41μM (Liu & Wang, 1997; Wang & Kurzer, 1998). (Hoffman, 1995) found genistein IC50 values of 12.6μM in the presence of no growth factors and an IC50 of 5.9μM in the presence of 10ng/ml IGF-1. Studies using BRDU incorporation to assess DNA synthesis have also shown biphasic effects in MCF-7 cells. (Hsu et al., 1999) found that in MCF-7 cells, biochanin A (the precursor to genistein) concentrations of less than 10μg/ml increased DNA synthesis by up to 280% of the control while greater than 30μg/ml significantly reduced DNA synthesis with an IC50 value of 40μg/ml.

The biphasic effects on DNA synthesis caused by increasing concentrations of isoflavones in MCF-7 cells agrees with results from other studies done in variying assays including:

- direct cell counting with a Coulter Counter (Dampier et al., 2001; Schmitt et al., 2001)
- cell doubling time after 7 days incubation (Matsumura et al., 2005)
- cell number-colorimetric MTT assay (Le Bail et al., 1998)
- protein content-sulphorhodamine colorimetric assay (Sathyamoorthy & Wang, 1997; Sathyamoorthy et al., 1994; Wang et al., 1996)
- DNA content- diphenylamine assay (Fioravanti et al., 1998)
- DNA content- Krishans propidium iodide stain (Zava & Duwe, 1997)
- DNA content- Hoeshst reagent (Hsieh et al., 1998).

Studies examing MCF-7 cell number, found genistein IC50 values of 36μM (Monti & Sinha, 1994), 39μM (Peterson & Barnes, 1991) and 24-38μM (Matsukawa et al., 1993). Our comparatively high IC50 values could well be lowered if concentratiosn beween 10 and 100μM had been examined, as inhibition appears to increase steeply in the cells between these concentrations. A recent paper examining DNA synthesis using the BRDU assay and expression of ERα/ERβ in MCF-7 and T47D cells (Power & Thompson, 2003) found biphasic effects in both cell lines, with maximal proliferation of ~250% control at 10μM genistein which then decreased at 100μM in MCF-7 cells. Western blot analysis showed that 1μM genistein caused a down-regulation of ERα while having no effect on ERα in T47D cells and an upregulation of ERβ in both cell types. This pattern of differential regulation of the ERs between the two cell lines was also seen with oestradiol stimulation at 1nM. This suggests that proliferation of DNA synthesis by genistein at this concentration is using similar

mechanisms to that of oestradiol, while the inhibitory action at higher concentrations appears to be only partially mediated through ER dependent pathways (Chen et al., 2003).

In the ERα negative MDA-MB 231 cells, isoflavones did not cause proliferation of DNA synthesis at lower concentrations but showed significant inhibition at higher concentrations. The IC_{50} values of 14 μM, 113μM and 15μM for genistein, daidzein and equol respectively are consistent with findings reported by other studies in other assay types.

The IC_{50} for DNA synthesis, as determined through thymidine incorporation, for genistein and daidzein, were 27μM and 81μM respectively (Liu & Wang, 1997). Assays of cell number in MDA-231 cells found IC_{50} of 15μM for genistein (Monti & Sinha, 1994) and in MDA-468 cells 24μM and 134μM for genistein and daidzein, respectively (Peterson & Barnes, 1991). Genistein concentrations >1μM markedly inhibited MDA-468 cell growth measured as DNA content (Zava & Duwe, 1997) and genistein concentrations of 0.01-10μM were inhibitory of DNA conentent in MDA-231 cells (Wang et al., 1996). (Fioravanti et al., 1998) also found that > 1μM genistein inhibited cell growth as measured through DNA content with 5μM giving 20% inhibition and significant counteraction of IGF-1 stimulation ($p<0.01$). A 10μM concentration of genistein significantly inhibited cell number in MDA-MB 231 cells (Dampier et al., 2001). (Tanos et al., 2002) also showed inhibition of MDA-MB 231 cells between the genistein concentrations of 0.4-4μM with 54.6% of cell growth at 4μM using methylene blue staining method. (Shao et al., 1998a) found a genistein IC50 for cell growth of 20μg/ml (74μM) for both MCF-7 and MDA-231 cells, while (Schmitt et al., 2001) showed that concentrations of Daidzein and Equol >10μM inhibited cell proliferation in this cell line.

6.4.2 Interactions between Isoflavones and oestradiol

In the ERα positive MCF-7 cells, the three isoflavones studied at 10μM concentrations all significantly inhibited 1nM oestradiol induced synthesis. These results agree with other studies which have shown that, like the biphasic effects seen when isoflavones are given on their own, low concentrations enhance oestradiol proliferation and high concentrations (≥10μM) inhibit oestradiol induced proliferation (Harris et al., 2005; Le Bail et al., 1998; Power & Thompson, 2003; Wang & Kurzer, 1998). (Kuiper et al., 1998) investigated the induction of gene expression by a combination of oestradiol with genistein and found that low concentrations of 10^{-7}M and 10^{-6}M had additive effects on the activation of transcription in both ERα and ERβ.

At higher concentrations (Zava & Duwe, 1997) found that the pronounced growth-inhibitory actions of genistein over the 10-20μM concentration were not modified by 0.3nM E_2. (So et al., 1997) showed that inhibition in cell growth caused by 18.5μM genistein in MCF-7 cells was restored by 100nM oestradiol, while (Schmitt et al., 2001) also found that 1-10μM of daidzein and equol in combination with 100pM oestradiol reduced cell proliferation compared to the treatment with oestradiol alone. (Matsumura et al., 2005) also showed an inhibition of oestradiol stimulated growth after 7 days when combined with genistein and equol concentrations of 10^{-5}M.

(Power & Thompson, 2003) found that when genistein was combined with 1nM oestradiol and compared to cells treated with oestradiol alone it significantly decreased cell proliferation at concentrations ≥ 10μM in MCF-7 and ≥1μM in T47D cells. They found that ERβ was upregulated when 1μM genistein or 1nM oestradiol was given alone but not when given in combination.

These results could be explained by a competitive reaction of the test compounds with oestradiol for the receptor subtypes, as genistein competes strongly with oestradiol, with (Kuiper et al., 1998) showing that at concentrations of 1μM, genistein has a greater oestrogenic potency (in the form of trans activation activity for ERα and Erβ).

(Zava & Duwe, 1997) suggests that genistein, while mediating its actions via ER as an estrogen agonist, apparently also cross talks with other ER-independent cellular mechanisms at higher concentrations to inhibit cell proliferation induced by genistein through ER pathways. However, whether this activity may potentially be due to extragenomic actions of these ligands as concluded by (Harris et al., 2005) such as antioxidant activity G2M arrest of the cell cycle and/or possibly the result of ERβ exerting an inhibitory action on ERα mediated gene expression, still remains to be elucidated (Matthews & Gustafsson, 2003). A recent study by (Chen et al., 2003) showed that genistein at high concentrations down regulates the SRF response element, which is involved in both ER and IGF-R mediated pathways (Duan et al., 2001). However this occurred in the presence of a pure antiestrogen ICI 182, 780 which suggests this is not purely an ER dependent mechanism.

In MDA-231 cells however, the addition of 1nM oestradiol appeared to rescue the cells slightly from the inhibitory effects of the isoflavones with an increase in synthesis of 10%, 7.9% and 11% of gensitein, daidzein and equol respectively. The reason for this may be due to competitive binding for ERβ, however the combinatory effects of isoflavones with oestradiol have not been investigated by others in ER negative cell lines and thus requires further investigation.

6.4.3 Interactions between oestradiol and isoflavones with Tamoxifen

In MCF-7 cells, tamoxifen inhibited DNA synthesis and this inhibition was significant from 0.1μM onwards with an IC50 of 1.2μM. These results agree with other studies in this cell line (Faye et al., 1987; Jordan et al., 1985; Power & Thompson, 2003; Tanos et al., 2002; Taylor et al., 1984).

Concentrations of 1μM tamoxifen also inhibited 1nM oestradiol induced synthesis by 28% ($P<0.01$). This is consistent with results by (Wang & Kurzer, 1998) who showed a decrease in DNA synthesis of 23% at these concentrations. Other studies have also shown inhibitory effects of tamoxifen on oestradiol induced proliferation at varying concentrations (Power & Thompson, 2003; Schmitt et al., 2001) The combination of 1μM tamoxifen and 1nM oestradiol induced the same pattern of ERα/β regulation as tamoxifen alone in MCF-7 cells (Power & Thompson, 2003). Tamoxifen has been showen to counteract oestradiol induced synthesis in part by binding and causing transcriptional activation of ERβ (Paech et al., 1997).

Conversely, in this study the antiestrogenic actions of 1μM tamoxifen which inhibited DNA synthesis to 60% when given alone or by 28% when given with oestradiol, was completely counteracted when combined with 10μM genistein and daidzein such that DNA synthesis actually increased by 22% and 15% respectively ($P<0.05$) compared with isoflavones given alone.

This is consistent with findings by (Wang & Kurzer, 1998) who showed that tamoxifen at concentrations of 0.1-1μM enhanced the oestrogenic effects of low concentrations (0.1-10μM) of gensitein.

In contrast, others have found that although genistein may override the growth inhibitory actions of tamoxifen at concentrations of 10nM-1μM, tamoxifen still reduces genistein induced protein content when compared with genistein given

alone (Zava & Duwe, 1997). While at 0.01μM tamoxifen can further inhibit cell proliferation of 0-37μM genistein concentrations (Tanos et al., 2002) and decrease expression of genistein stimulated pS2 mRNA expression (Wang et al., 1996).

It is interesting to note that the increase we saw in DNA synthesis with genistein and daidzein was not seen when tamoxifen was combined with 10μM equol and the reasons for this requires further investigation. Studies by (Sathyamoorthy & Wang, 1997) have found equol to be 100 fold more potent than daidzein in stimulating pS2 expression in MCF-7 cells. They found that 1μM tamoxifen was able to completely inhibit pS2 expression by 0.1μM daidzein but could only partially inhibit equol stimulation (55%) at this concentration. Equol was far more effective at inhibiting the binding of oestradiol to ER with 0.05μM resulting in 50% inhibition compared to 20% for Daidzein at the same concentration. A study by (Magee et al., 2006) has also found that R and S-equol enantiomers have different biological properties with the Racemic equol responsible for antioxidant effects preventing DNA damage in MCF-7 cells.

In the ERα negative MDA 231 cells, the opposite was true. The addition of genistein and equol caused a further inhibtion of 11% and 22% respectively than antiproliferative effects seen with 1μM tamoxifen alone (74.5%) or with 1nM Estradiol alone (68.2%). This synergistic inhibition has also been reported by (Shen et al., 1999) who found that 5μM tamoxifen and genistein decreased cell inhibition to 44% of the control in MDA-435 cells and agrees with results by (Schmitt et al., 2001; Tanos et al., 2002). An explanation for the observed synergistic anti-proliferative action may be the result of tamoxifen and genistein attacking different targets in signal transduction at different phases in the cell cycle (G1 and G2M respectively)(Shen et al., 1999). Why this synergism was

not seen in MCF-7 cells is a matter for discussion, however, it would seem that the presence of ERα complicates the non-estrogenic actions of these compounds. We should note that we did not see this synergism in inhibition with daidzein and this may be explained by some reports which have showen that daidzein does not bind with any great affinity to ERβ compared with genistein and equol (Kuiper et al., 1998; Matsumura et al., 2005).

6.4.4 Interatctions between isoflavones and Tamoxifen with IGF-1

Early studies showed that genistein inhibited tyrosine kinase activity of growth factor receptors EGF, PDGF, insulin and IGF-1 (Akiyama et al., 1987; Akiyama & Ogawara, 1991; Kawase et al., 1995). Studies have also shown that antiestrogens can inhibit the IGF-1 induced transcriptional activation of the ER (Clayton et al., 1997; Lee et al., 1997a; Newton et al., 1994; Westley & May, 1994) with increasing substantiation of interactions between the ER and IGF-1R pathways in ERα positive breast cancer cells (Cascio et al., 2007; Kato et al., 2000; Martin & Stoica, 2002; Mendez et al., 2003; Surmacz & Bartucci, 2004). To investigate these effects, DNA synthesis was measued in both MCF-7 cells and MDA-231 cells after incubation with isoflavones in the presence of IGF-1. The stimulation of IGF-1 in MCF-7 cells was reduced by the addition of all three isoflavones and tamoxifen. However, although reductions of 29%, 16% and 28% were seen for genistein, daidzein and tamoxifen respectively, only the equol reduction of 46.2% ($P<0.01$) was significant.

These results compare with two previous studies examining genistein inhibition of IGF-1 or insulin stimulated growth. (Hoffman, 1995) found an IC50 value of 5.9μM genistein for inhibition of 10 ng/ml IGF-1 stimulated DNA synthesis of MCF-7 cells. (Fioravanti et al., 1998) found that 5μM genistein significantly

counteracted 10ng/ml IGF-1 stimulation of DNA content ($p<0.001$). (Pagliacci et al., 1994) also found that 100μM genistein inhibited insulin induced tyrosine phosphorylation and a strong increase in the percentage of S phase cells. (Wang & Kurzer, 1998) found that 0.1μM and 1.0μM genistein significantly increased insulin induced DNA synthesis, while 10μM genistein inhibited DNA synthesis induced by 1-2 μg/ml insulin and ≥ 25μM genistein inhibited DNA synthesis induced by 0.5-2μg/ml insulin ($p<0.01$). (Panno et al., 1996) found that >37μM Genistein inhibited insulin induced growth in MCF-7 cells. However, to our knowledge there are no other studies that have examined the effects of daidzein and equol on IGF-1 induced DNA synthesis in MCF-7 cells.

The tamoxifen reductions in IGF-1 induced DNA synthesis also agree with other studies, which have shown that 1μM tamoxifen can significantly inhibit IGF-1 induced proliferation in ERα positive cell lines by inhibiting IGF-1 mediated transcriptional activation of the endogenous ER (Lee et al., 1997a), down regulate IGF-1 induced tyrosine phosphorylaltion of IGF-1R and inhibit IRS-1 (Guvakova & Surmacz, 1997). Antiestrogens such as tamoxifen, have also been shown to down regulate the expression of IGF binding proteins (Winston et al., 1994). Certainly the interaction between oestrogens and the IGF-1 signaling pathway could explain some of the isoflavone biphasic effects seen in ER postive cell lines and possibly the synergistic effects seen with combinations of tamoxifen and isoflavones.

Oestradiol has been showen to upregulate IGF-1 receptor mRNA expression (Stewart et al., 1990), enhance IGF-1 signalling through IRS-1 expression (Lee et al., 1999; Surmacz & Bartucci, 2004), activate MAP Kinase pathway (Migliaccio et al., 1996) and prevent apoptosis through a synergistic increase in Akt protein activity (Ahmad et al., 1999), with studies by (Martin & Stoica, 2002),

showing that IGF-1 regulates both the expression and the activity of ERα in part through the phos-phatidylinositol-3-kinase/Akt pathway. More recent studies by (Cascio et al., 2007) show that both oestradiol and IGF-1 differentially regulate ER transcription at ERE and AP-1 sites. A recent study by (Chen & Wong, 2004) has looked directly at the effects of genistein at low concentrations (1μM) on the IGF-1 signalling pathway. They found that 1μM genistein like 10nM oestradiol increases protein, mRNA expression and enhances tyrosine phosphorylation of the IGF-1R and downstream signalling protein IRS-1. These effects were completely counteracted by the antiestrogens ICI 182,780 and tamoxifen, sugesting that ER and more specifically the AF-2 domain of the ER is most likely involved in mediating these effects. The author concluded that the IGF signalling pathway is at least partly responsible for the growth promoting effects of genistein in MCF-7 cells at low concentrations. However, further investigation should be done to determine if higher doses of genistein cause inhibition of the IGF-1 signalling pathway or mediate their antiproliferative actions through other mechanisms. It would also be valuable to conduct these experiments with other isoflavones and antiestrogens in combination and in cell lines that do not express ERα, such as MDA-231 cells, to understand more fully the mechanisms involved.

In MDA-231 cells the addition of IGF-1 actually reduced the ability of genistein, daidzein and equol to inhibit DNA synthesis alone by 11%, 20% and 17% respectively ($P<0.05$). Genistein inhibited IGF-1 stimulated growth by 22% compared with IGF-1 alone, which is similar to the findings of (Fioravanti et al., 1998) who found that 5μM genistein was able to inhibit IGF-1 stimulated DNA content by 20%. However, we did not see this with tamoxifen, which showed no

difference in the amount of DNA inhibition with or without IGF-1 at the concentrations used.

The synergistic increase in DNA synthesis when oestradiol and IGF-1 were combined ($P<0.01$) is in line with studies that have looked at these effects in ER α positive cell lines (Stewart et al., 1990) but have not been investigated with the MDA-231 ERα negative cell line.

These results suggest that the isoflavones are working differently to tamoxifen in that part of the isoflavone inhibitory action (possibly through ER-β) is counteracted by the addition of IGF-1 and stimulation of the IGF-1R mediated pathway. To our knowledge there are as yet no published studies that have examined the interactions of daidzein and equol with IGF-1 in MCF-7 or MDA-231 ERα negative cell lines.

6.5 Conclusion

In conclusion, the most plausible explanation for the biphasic effects of these isoflavone compounds in MCF-7 cells is that, while mediating their actions via ER as oestrogen agonists, they can also cross talk with the IGF-1 signalling pathway using this and other ER independent cellular mechanisms at higher concentrations to inhibit cell proliferation. Certainly the presence of ERα is not required for the isoflavones to inhibit tumor cell growth as seen by the results in the MDA-231 cells. The isoflavones themselves also appear to have different qualities with the metabolite equol showing greater inhibition than the parent compound daidzein in both cell lines.

Isoflavones do appear to be inhibitory in concentrations ≥10μM and the synergism of tamoxifen especially with equol in the ERα negative MDA-231 cells is certainly worth further investigation. The clinical concern is whether

physiological concentrations could be sustainable at these levels and the evidence for this is conflicting. Reports also indicate that not everyone produces equol as a metabolite and thus it may need to be provided in supplement form. Finally, to our knowledge, this is the first report to assess the effect of isoflavones daidzein and equol on IGF-1 induced DNA synthesis in MCF-7 and MDA-231 cells and although the results do not provide direct biochemical evidence for antagonism of the IGF-1R signalling pathway they do show that biological effects of IGF-1 on MCF-7 and MDA-231 cells are counteracted by isoflavones, especially equol.

CHAPTER SEVEN – CONCLUSION

The potential role for isoflavones in the prevention of breast cancer is yet to be fully established, whilst the mechanisms involved also remain to be further elucidated.

Certainly the intervention studies conducted here and others published to date show that these compounds have only moderate effects on hormonal status and little impact in lowering endogenous levels of IGF-1, unlike pharmacological compounds such as tamoxifen. Further well-designed and controlled intervention studies using isolated isoflavones are required to determine any differences when compared with soy protein, which appears to increase circulating levels of IGF-1 and its binding proteins.

The heterogeneity of response to isoflavone exposure, which depends on gut microflora activity, necessitates an increase in the numbers of subjects required for an adequately powered study (Bingham et al., 1998) as increasing evidence suggests that equol producers may have greater clinical benefits. Differences in lipid and antioxidant status in equol excretors versus non-excretors in our one month supplementation study would appear to support this. The effect of habitual dietary intake on the proportion of metabolites excreted remains to be determined. Further studies are also needed to determine the amounts of equol ingested directly from meat and dairy products as opposed to created through a conducive microflora environment. It may also be of interest to study supplementation with isolated equol supplements to reduce study complications.

Certainly other dietary components may well be more effective in altering IGF-1 status and this requires further investigation. In particular it may be more effective to intervene with more global dietary changes such as a low fat high fibre diet as seen with recent large long term intervention studies, rather than supplementing with isolated compounds.

With isoflavone effects on endogenous levels of IGF apparently minimal it may be that their benefits are more localised to the breast. *In vitro* results from our studies in breast cancer cell lines and others suggest that isoflavones, especially equol, may be useful in establishing the pharmacological qualities of compounds needed to overcome resistance to antiestrogens which may be occurring through the IGF-1R and ER cross-talking mechanisms. Whether or not the isoflavones' greater affinity for ERβ may be conferring an ability to act synergistically with tamoxifen remains to be examined. Further investigation should also be done to examine the isoflavones' ability to increase signal transduction of the IGF-1R pathway, which occurs at low concentrations and could well explain the biphasic effects seen with these compounds.

Certainly the ability of isoflavones to increase proliferation in ERα positive breast cancer cell lines at lower concentrations should not be ignored, with current opinion that breast cancer survivors and those at risk should avoid excess consumption of these "weak estrogenic compounds" until further research has been carried out. Further studies should be done to fully establish concentrations at the cellular level in populations who already consume soy isoflavones, while future intervention studies in subpopulations at higher risk of breast cancer, and in those with breast cancer, may give us the answers required.

Emerging animal study evidence suggests that short-term neonatal and pre-pubertal exposure to dietary isoflavones decreases carcinogen-induced breast cancer by increasing the proportion of differentiated cells in the mammary gland (Lamartiniere et al., 1995; Miller et al., 1994). This would support a concept that the protective effect of the traditional Asian diet occurs early in life, (Colditz & Frazier, 1995) and may explain why epidemiological studies and intervention studies, focussing on adult intake, have to date been unimpressive. Future prospective studies examining outcomes of adults who received soy based formula as infants may be of interest although a recent study comparing infants and children on cows' milk vs soya formula suggested no lasting effect of early life isoflavone exposure on isoflavone metabolism (Hoey et al., 2004). Finally, further epidemiological evidence coming from large prospective studies should point to nutritional links with breast cancer but may not be able to disentangle the beneficial effects of higher fibre/lignans/flaxseed or low fat diets that go hand in hand with soy consumption and an overall healthier diet pattern. Long term larger intervention studies with isolated isoflavones including equol, in populations at higher risk of developing breast cancer, will hopefully add to the evidence base. Certainly IGF-1 and its signalling pathway appear to be prominent targets for future treatments given they have such an important role in protecting tumour cells from apoptosis. Lowering physiological levels and expression of the IGF-1R and its ligand IGF-1 also appear to be important components in the prevention of metastasis, with antiestrogenic treatment influencing levels *in vivo*. Just how effective natural antiestrogenic compounds like isoflavones are in lowering IGF-1 production, influencing the signalling pathway and ultimately breast cancer risk remains to be determined and certainly requires further research.

APPENDIX

Table 5.A.1: Correlations of equol excretion with serum lipids, serum antioxidants, dietary intake (pre and post-menopausal women) and anthropometric measurements.

	Equol (ug/day) P(1-3)	Equol (ug/day) P(26-28)	Equol (ug/day) S(1-3)	Equol (ug/day) S(26-28)
LDL P(1-3)				R=0.487 P=0.034 N=19
LDL S(26-28)				R=0.571 P=0.011 N=19
MDA P(1-3)				R=0.676 P=0.001 N=19
CHOL P(1-3)				R=0.579 P=0.009 N=19
CHOL S(26-28)				R=0.528 P=0.020 N=19
TRIG P(1-3)				R=0.685 P=0.001 N=19
TRIG S(1-3)		R=0.486 P=0.030 N=20		R=0.577 P=0.010 N=19
RET P(1-3)				R=0.487 P=0.034 N=19
g-TOC P(1-3)				R=0.873 P=0.000 N=19
g-TOC P(26-28)		R=0.469 P=0.037 N=20		
a-TOC P(1-3)				R=0.630 P=0.004 N=19
a-TOC S(1-3)		R=0.475 P=0.035 N=20		
LYCO P(1-3)			R=0.593 P=0.007 N=19	
Iodine PFFQ				R=0.590 P=0.016 N=16
%Alcohol PFFQ	R=0.823 P=0.000 N=17			
Carbo SFFQ			R=0.498 P=0.036 N=18	
Energy Kcal SFFQ			R=0.548 P=0.019 N=18	

Starch SFFQ			R=0.639 P=0.004 N=18	
Sodium SFFQ			R=0.500 P=0.035 N=18	
Iron SFFQ			R=0.548 P=0.019 N=18	
%Protein SFFQ		R=0.555 P=0.017 N=18		
%alcohol SFFQ	R=0.815 P=0.000 N=17			
Water PFD			R=-0.583 P=0.014 N=17	
Fat PFD	R=0.552 P=0.022 N=17			
Energy Kcal PFD	R=0.508 P=0.037 N=17			
PUFA PFD	R=0.528 P=0.029 N=17			R=0.551 P=0.022 N=17
Chol PFD	R=0.615 P=0.009 N=17			
Starch PFD			R=0.518 P=0.033 N=17	
Selenium PFD		R=0.482 P=0.050 N=17	R=0.576 P=0.016 N=17	
Vit D PFD				R=0.516 P=0.034 N=17
Niacin PFD	R=0.497 P=0.042 N=17			
%alcohol PFD	R=0.508 P=0.037 N=17			
Vit D SFD				R=0.577 P=0.019 N=16
% alcohol SFD	R=0.748 P=0.001 N=17			
Weight				R=0.503 P=0.028 N=19
Waist				R=0.588 P=0.013 N=17
BMI				R=0.517 P=0.023 N=19

Data compared using pearson correlation and presented (r ,P)

Table 5.A.2: Correlations of Enterolactone excretion with serum lipids, serum antioxidants and dietary intake (pre and postmenopausal women).

	Enterolactone P(1-3)	Enterolactone P(26-28)	Enterolactone S(1-3)	Enterolactone S(26-28)
b-Cryp P(26-28)			R=0.506 P=0.019 N=21	R=0.554 P=0.009 N=21
a-Caro P(1-3)			R=0.531 P=0.013 N=21	
a-Caro P(26-28)				R=0.580 P=0.006 N=21
a-Caro S(1-3)	R=0.573 P=0.007 N=21	R=0.530 P=0.014 N=21		R=0.644 P=0.002 N=21
a-Caro S(26-28)		R=0.453 P=0.039 N=21	R=0.553 P=0.009 N=21	R=0.586 P=0.005 N=21
b-Caro P(1-3)			R=0.440 P=0.046 N=21	
b-Caro P(26-28)				R=0.466 P=0.033 N=21
b-Caro S(1-3)	R=0.501 P=0.021 N=21	R=0.467 P=0.033 N=21		R=0.620 P=0.003 N=21
b-Caro S(26-28)			R=0.484 P=0.026 N=21	R=0.441 P=0.045 N=21
Manganese PFD			R=0.487 P=0.035 N=19	
Carotene PFD			R=0.459 P=0.048 N=19	
Zinc SFD			R=0.546 P=0.024 N=17	
Retinol SFD	R=0.579 P=0.009 N=19	R=0.511 P=0.030 N=18		R=0.517 P=0.028 N=18
Thiamin SFD			R=0.504 P=0.039 N=17	
Riboflavin SFD			R=0.518 P=0.033 N=17	

Data compared using pearson correlation and presented (r ,P)

Table 5.A.3: Correlations of Enterodiol excretion with serum lipids, serum antioxidants and dietary intake (pre and postmenopausal women)

	Enterodiol P(1-3)	Enterodiol P(26-28)	Enterodiol S(1-3)	Enterodiol S(26-28)
MDA P(1-3)			R=0.437 P=0.048 N=21	
Chol S(26-28)		R=0.436 P=0.048 N=21		
Vit C P(1-3)			R=0.483 P=0.027 N=21	
g-TOC P(1-3)	R=0.581 P=0.006 N=21	R=0.555 P=0.009 N=21		R=0.706 P=0.000 N=21
a-TOC P(1-3)		R=0.450 P=0.041 N=21		
Zeax P(1-3)	R=0.457 P=0.037 N=21	R=0.515 P=0.017 N=21		
Iodine PFFQ				R=0.611 P=0.007 N=18
Protein PFD			R=0.552 P=0.014 N=19	
Carbohydrate PFD		R=0.502 P=0.034 N=18		
Energy Kcal PFD			R=0.482 P=0.037 N=19	
Nitrogen PFD			R=0.553 P=0.014 N=19	
PUFA PFD	R=0.471 P=0.042 N=19			
Chol PFD			R=0.501 P=0.029 N=19	
Sugar PFD		R=0.482 P=0.043 N=18		
Fibre1 PFD		R=0.578 P=0.012 N=18		
Fibre2 PFD		R=0.594 P=0.009 N=18		
Potassium PFD		R=0.692 P=0.001 N=18		
Magnesium PFD		R=0.605 P=0.008 N=18		
Phosphorous PFD		R=0.515 P=0.029 N=18		
Iron PFD		R=0.473		

		P=0.041 N=19		
Carotene PFD		R=0.547 P=0.019 N=18		
Vitamin D PFD	R=0.659 P=0.002 N=19	R=0.646 P=0.004 N=18		
Vitamin E PFD	R=0.610 P=0.006 N=19			
Niacin PFD			R=0.533 P=0.019 N=19	
Tryptophan PFD			R=0.543 P=0.016 N=19	
Vit B6 PFD		R=0.648 P=0.004 N=18		
Vit B12 PFD		R=0.682 P=0.002 N=18		
Pantothenic PFD		R=0.469 P=0.050 N=18	R=0.634 P=0.004 N=19	
Biotin PFD		R=0.508 P=0.031 N=18		
Retinol SFD	R=0.538 P=0.017 N=19			
Vit D SFD		R=0.644 P=0.004 N=18		
%Fat SFD	R=0.511 P=0.025 N=19			
%Alcohol SFD			R=0.519 P=0.033 N=17	

Data compared using pearson correlation and presented (r ,P)

Table 5.A.4: Correlations of Genistein excretion with serum lipids, serum antioxidants and dietary intake (pre and post-menopausal women)

	Gensitein P(1-3)	Genistein P(26-28)	Gensitein S(1-3)	Genistein S(26-28)
HDL P(26-28)				R=-0.479 P=0.038 N=19
TRIG P(1-3)				R=0.525 P=0.021 N=19
TRIG P(26-28)				R=0.457 P=0.049 N=19
TRIG S(26-28				R=0.532 P=0.019 N=19
Retin P(1-3)	R=0.486 P=0.025 N=21			
Vit C S(26-28)				R=-0.482 P=0.043 N=18
Lutein P(1-3)			R=-0.451 P=0.046 N=20	
Lutein P(26-28)	R=0.492 P=0.023 N=21			
Lutein S(1-3)	R=0.544 P=0.011 N=21			
Lutein S(26-28)	R=0.501 P=0.021 N=21			
Zeax P(26-28)			R=-0.463 P=0.040 N=20	
Zeax S(26-28)				R=-0.486 P=0.035 N=19
BCryp P(1-3)	R=0.464 P=0.034 N=21			
Bcryp P(26-28)	R=0.467 P=0.033 N=21			
a-Caro P(1-3)	R=0.439 P=0.046 N=21		R=-0.542 P=0.013 N=20	
b-Caro P(1-3)			R=-0.630 P=0.003 N=20	
b-Caro S(26-28)			R=-0.446 P=0.049 N=20	
Lyco P(1-3)			R=-0.658 P=0.002 N=20	
Carotene PFFQ	R=0.527 P=0.020 N=19			
%Alcohol PFFQ		R=0.687		

		P=0.002 N=18		
Water PFD				R=0.524 P=0.026 N=18
Iodine PFD				R=0.483 P=0.043 N=18
Niacin PFD		R=0.560 P=0.016 N=18		
Vitamin C PFD	R=0.517 P=0.023 N=19			
%Alcohol PFD		R=0.494 P=0.037 N=18		
Carotene SFD				R=-0.526 P=0.030 N=17
Thiamin SFD	R=0.525 P=0.021 N=19			
Riboflavin SFD	R=0.462 P=0.046 N=19			
%Protein SFD		R=-0.478 P=0.045 N=18	R=-0.501 P=0.041 N=17	
%Alcohol SFD		R=0.540 P=0.021 N=18		
Age				R=0.512 P=0.025 N=19
Weight				R=0.648 P=0.003 N=19
Waist				R=0.579 P=0.015 N=17
Hip				R=0.659 P=0.004 N=17
BMI				R=0.574 P=0.010 N=19

Data compared using pearson correlation and presented (r ,P)

Table 5.A.5: Correlations of Daidzein excretion with serum lipids, serum antioxidants, dietary intake and anthropometric measurements (pre and postmenopausal women)

	Daidzein P(1-3)	Daidzein P(26-28)	Daidzein S(1-3)	Daidzein S(26-28)
RETIN P(26-28)		R=0.449 P=0.041 N=21		
G-Toc S(1-3)			R=0.498 P=0.022 N=21	
G-Toc S(26-28)			R=0.485 P=0.026 N=21	
A-Toc S(1-3)		R=0.464 P=0.034 N=21		
b-Cryp P(26-28)			R=0.445 P=0.043 N=21	
b-Cryp S(26-28)			R=0.539 P=0.012 N=21	
a-Caro P(26-28)		R=0.469 P=0.032 N=21		
Carbo PFD				R=0.710 P=0.001 N=19
Energy Kcal PFD				R=0.548 P=0.015 N=19
Starch PFD				R=0.589 P=0.008 N=19
Sugar PFD				R=0.54- P=0.017 N=19
Fibre 1 PFD		R=0.550 P=0.018 N=18	R=0.478 P=0.039 N=19	R=0.811 P=0.000 N=19
Fibre 2 PFD		R=0.627 P=0.005 N=18	R=0.512 P=0.025 N=19	R=0.689 P=0.001 N=19
Potassium PFD		R=0.595 P=0.009 N=18		R=0.574 P=0.010 N=19
Magnesium PFD		R=0.584 P=0.011 N=18		R=0.620 P=0.005 N=19
Phosphorous PFD				R=0.479 P=0.038 N=19
Iron PFD		R=0.527 P=0.025 N=18		R=0.565 P=0.012 N=19
Copper PFD		R=0.619 P=0.006 N=18		
Zinc PFD		R=0.558 P=0.016		R=0.493 P=0.032

		N=18		N=19
Manganese PFD	R=0.604 P=0.006 N=19	R=0.780 P=0.000 N=18	R=0.70 P=0.001 N=19	R=0.630 P=0.004 N=19
Riboflavin PFD		R=0.536 P=0.022 N=18		
Nicain PFD		R=0.522 P=0.026 N=18		
VitB6 PFD		R=0.610 P=0.007 N=18		R=0.560 P=0.013 N=19
Vit B12 PFD		R=0.607 P=0.001 N=18		
Folate PFD		R=0.562 P=0.015 N=18	R=0.528 P=0.020 N=19	
Pantothenic PFD		R=0.692 P=0.001 N=18	R=0.516 P=0.024 N=19	R=0.581 P=0.009 N=19
Biotin PFD		R=0.557 P=0.016 N=18	R=0.508 P=0.026 N=19	
Water SFD			R=0.543 P=0.024 N=17	
Protein SFD			R=0.577 P=0.015 N=17	
Fat SFD		R=0.577 P=0.012 N=18		
Cabo SFD		R=0.474 P=0.047 N=18		R=0.584 P=0.011 N=18
Energy Kcal SFD		R=0.549 P=0.018 N=18		R=0.512 P=0.030 N=18
Nitrogen SFD			R=0.577 P=0.015 N=17	
SFA SFD		R=0.468 P=0.050 N=18		
MUFA SFD		R=0.588 P=0.010 N=18		
Sugar SFD				R=0.543 P=0.020 N=18
Fibre 1 SFD		R=0.557 P=0.016 N=18		R=0.482 P=0.043 N=18
Fibre 2 SFD		R=0.523 P=0.026 N=18	R=0.513 P=0.035 N=17	R=0.552 P=0.018 N=18
Magnesium SFD		R=0.526 P=0.025 N=18	R=0.653 P=0.004 N=17	R=0.534 P=0.022 N=18
Phosphorous			R=0.606	R=0.479

SFD			P=0.010 N=17	P=0.044 N=18
Iron SFD		R=0.667 P=0.003 N=18	R=0.644 P=0.005 N=17	R=0.559 P=0.016 N=18
Copper SFD	R=0.544 P=0.016 N=19	R=0.616 P=0.007 N=18	R=0.574 P=0.016 N=17	
Zinc SFD		R=0.485 P=0.041 N=18	R=0.670 P=0.003 N=17	
Manganese SFD	R=0.605 P=0.006 N=19	R=0.683 P=0.002 N=18	R=0.745 P=0.001 N=17	
Selenium SFD		R=0.621 P=0.006 N=18	R=0.635 P=0.006 N=17	R=0.668 P=0.002 N=18
Retinol SFD		R=0.542 P=0.020 N=18		
Vit D SFD				R=0.493 P=0.038 N=18
Thiamin SFD		R=0.614 P=0.007 N=18	R=0.541 P=0.025 N=17	
Riboflavin SFD			R=0.698 P=0.002 N=17	
Niacin SFD			R=0.649 P=0.005 N=17	R=0.551 P=0.018 N=18
Tryptophan SFD			R=0.555 P=0.021 N=17	
Vit B6 SFD			R=0.541 P=0.025 N=17	R=0.471 P=0.048 N=18
Vit B12 SFD		R=0.632 P=0.005 N=18	R=0.667 P=0.003 N=17	R=0.778 P=0.000 N=18
Folate SFD			R=0.563 P=0.019 N=17	
Pantothenic SFD	R=0.461 P=0.047 N=19	R=0.533 P=0.023 N=18	R=0.611 P=0.009 N=17	
Biotin SFD	R=0.522 P=0.022 N=19			
Age			R=0.495 P=0.022 N=21	

Data compared using pearson correlation and presented (r ,P)

Table 5.A.6: Correlations of serum concentrations of Isoflavones (ug/day) in Pre-menopausal women with Serum IGF-1, BP-3 and BP-1 throughout the menstrual cycle.

	Genistein Supp/post	Daidzein Supp/post	Equol Placeb/base	Equol Placeb/post	Equol Supp/base	Equol Supp/post
IGF1 Placebo(day1-3)					R=0.638 P=0.014 N=14	
IGF1 Placebo(day 6-8)	R=0.736 P=0.015 N=10					
IGF1 Placebo(day12-15)				R=-0.741 P=0.002 N=15		
IGF1 Placebo(day21-23)						R=0.548 P=0.042 N=14
IGF1 Supp(day1-3)		R=0.619 P=0.018 N=14	R=0.541 P=0.046 N=14			
IGF1 Supp(day12-15)		R=0.600 P=0.030 N=13				
BP3 Placebo(day6-8)				R=0.631 P=0.016 N=14		
BP1 Placebo(day1-3)			R=-0.566 P=0.035 N=14			
BP1 Supp(day12-15)			R=-0.597 P=0.031 N=13			

Data compared using pearson correlation and presented (r ,P)

Table 5.A.7: Correlations of serum concentrations of Enterolactone (ug/day) in pre-menopausal women with Serum IGF-1, BP-3 and BP-1 throughout the menstrual cycle.

	Enterolactone Placebo/Base	Enterolactone Placebo/post	Enterolactone Supp/post
BP-1 Placebo (day1-3)	R=0.590 P=0.026 N=14	R=0.505 P=0.055 N=15	R=0.655 P=0.011 N=14
BP-1 Placebo (day 6-8)	R=0.639 P=0.019 N=13	R=0.574 P=0.032 N=14	R=0.733 P=0.007 N=12
BP-1 Placebo (day 12-15)	R=0.528 P=0.052 N=14	R=0.454 P=0.089 N=15	R=0.716 P=0.004 N=14
BP-1 Placebo (day 21-23)	R=0.731 P=0.003 N=14	R=0.59 P=0.021 N=15	R=0.741 P=0.002 N=14
BP-1 Placebo (day 26-28)	R=0.664 P=0.010 N=14	R=0.525 N=0.045 N=15	R=0.709 P=0.005 N=14
BP-1 Supp (day 1-3)	R=0.686 P=0.007 N=14	R=0.604 P=0.017 N=15	R=0.811 P=0.000 N=14
BP-1 Supp (day 6-8)	R=0.652 P=0.011 N=14	R=0.550 P=0.034 N=15	R=0.775 P=0.001 N=14
BP-1 Supp (day 12-15)			R=0.673 P=0.012 N=13
BP-1 Supp (day 21-23)	R=0.684 P=0.007 N=14	R=0.578 P=0.024 N=15	R=0.782 P=0.001 N=14
BP-1 Supp (day 26-28)			R=0.710 P=0.004 N=14
IGF-1 Supp (day 26-28)			R=-0.670 P=0.009 N=14

Data compared using pearson correlation and presented (r ,P)

Table 5.A.8: Correlations of serum concentrations of Isoflavones (ug/day) in post-menopausal women with Serum IGF-1, BP-3 and BP-1.

	Genistein Placebo base	Genistein Placebo post	Genistein Supp post	Daidzein Placebo base	Daidzein Placebo post	Daidzein Supp post	Equol Placebo Post	Equol Supp Base	Equol Supp Post	Enterelact Placebo base
IGF1 Placebo base						R=0.804 P=0.029 N=7				R=0.806 P=0.029 N=7
IGF1 Placebo Post									R=0.917 P=0.028 N=5	
IGF1 Supp Base									R=0.983 P=0.003 N=5	R=0.791 P=0.034 N=7
IGF1 Supp Post					R=0.967 P=0.002 N=6	R=0.914 P=0.004 N=7				R=0.313 R=0.034 N=7
BP3 Placebo Base									R=0.989 P=0.001 N=5	
BP3 Placebo Post							R=0.898 P=0.038 N=5		R=0.912 P=0.031 N=5	
BP3 Supp Base									R=0.938 P=0.018 N=5	
BP3 Supp Post		R=0.920 P=0.009 N=6							R=0.933 P=0.020 N=5	
BP1 Placebo Base								R=0.931 P=0.021 N=5		
BP1 Supp Base							R=-0.940 P=0.017 N=5			
BP1 Supp Post	R=-0.776 P=0.040 N=7		R=0.804 P=0.029 N=7	R=-0.881 P=0.009 N=7						

Data compared using pearson correlation and presented (r ,P)

Table 5.A.9: Correlations of hormones with excretion of Isoflavones (ug/day) in post-menopausal women.

	Enterodiol P(1-3) Ug/day	Enterodiol P(26-28) Ug/day	Enterodiol S(1-3) Ug/day	Enterodiol S(26-28) Ug/day	Equol P(1-3) Ug/day	Equol S(1-3) Ug/day	Equol S(26-28) Ug/day	Daid S(26-28)	Genistein P(1-3) Ug/day	Genistein S(1-3) Ug/day	Enterolactone P(26-28) Ug/day	Enterolactone S(1-3) Ug/day
Progesterone P(1-3)											R=0.954 P=0.003 N=6	
Progesterone P(26-28)										R=0.919 P=0.003 N=7		
Progesterone S(26-28)	R=0.761 P=0.047 N=7						R=-0.970 P=0.006 N=5					
SHBG P(1-3)		R=0.834 P=0.039 N=6	R=0.875 P=0.010 N=7	R=0.836 P=0.019 N=7				R=0.906 P=0.005 N=7				
SHBG P(26-28)			R=0.809 P=0.027 N=7	R=0.837 P=0.019 N=7				R=0.852 P=0.015 N=7		R=-0.792 P=0.034 N=7		
SHBG S(1-3)				R=0.886 P=0.008 N=7						R=-0.841 P=0.018 N=7		
SHBG S(26-28)				R=0.817 P=0.025 N=7						R=-0.834 P=0.020 N=7		
Estrodiol S(1-3)						R=-0.890 P=0.043 N=5						
Estrodiol P(26-28)					R=0.939 P=0.018 N=5							
LH P(1-3)				R=0.713 P=0.042 N=7								
LH P(26-28)				R=0.787 P=0.036 N=7								
LH									R=0.797			R=0.760

S(1-3)									P=0.032 N=7			P=0.047 N=7
FSH P(1-3)				R=0.834 P=0.020 N=7						R=-0.797 P=0.032 N=7		
FSH S(1-3)										R=-0.839 P=0.018 N=7		
FSH S(26-28)				R=0.759 P=0.048 N=7						R=-0.763 P=0.046 N=7		

Data compared using pearson correlation and presented (*r* ,*P*)

Table 5.A.10: Correlations of hormones with excretion of Isoflavones (ug/day) in pre-menopausal women.

	Enterodiol P(1-3) Ug/day	Enterodiol P(26-28) Ug/day	Equol P(1-3) Ug/day	Equol P(26-28) Ug/day	Equol S(1-3) Ug/day	Daid P(1-3) Ug/day	Daid S(1-3) Ug/day	Daid S(26-28) Ug/day	Genistein P(1-3) Ug/day	Enterolactone P(1-3) Ug/day	Enterolactone P(26-28) Ug/day	Enterolactone S(1-3) Ug/day	Enterolactone S(26-28) Ug/day
Progesterone S(1-3)										R=0.653 P=0.011 N=14	R=0.617 P=0.014 N=		R=0.760 P=0.002 N=14
Progesterone S(12-15)													
Progesterone S(21-23)					R=0.620 P=0.018 N=14					R=0.588 P=0.034 N=13	R=0.589 P=0.027 N=14		R=0.669 P=0.012 N=13
Progesterone S(26-28)									R=-0.708 P=0.007 N=13			R=-0.598 P=0.031 N=13	
SHBG P(1-3)			r=-0.626 P=0.017 N=14										
SHBG S(1-3)			R=-0.535 P=0.049 N=14										
SHBG P(6-8)			R=-0.580 P=0.038 N=14							R=0.645 P=0.017 N=13	R=0.539 P=0.047 N=14		R=0.696 P=0.008 N=13
SHBG S(6-8)								R=-0.583 P=0.029 N=14					
SHBG P(12-15)			R=-0.654 P=0.011 N=14					R=-0.664 P=0.013 N=13					
SHBG S(12-15)								R=-0.599 P=0.030 N=14					
SHBG P(21-23)			R=-0.563 P=0.036 N=14										
SHBG S(21-23)			R=-0.578 P=0.03 N=14					R=-0.579 P=0.030 N=14					
SHBG P(26-28)			R=-0.613 P=0.020										R=0.583 P=0.029

			N=14										N=14
SHBG S(26-28)			R=-0.554 P=0.049 N=13					R=-0.534 P=0.049 N=14					
Estrodiol P(1-3)		R=0.564 P=0.028 N=15											
Estrodiol S(1-3)													
Estrodiol P(6-8)	R=0.796 P=0.001 N=13	R=0.539 P=0.047 N=14								R=0.950 P=0.000 N=13	R=0.907 P=0.000 N=14		R=0.959 P=0.000 N=12
Estrodiol S(6-8)		R=0.568 P=0.027 N=15											
Estrodiol P(26-28)		R=-0.619 P=0.014 N=15											
LH P(1-3)							R=0.564 P=0.036 N=14						
LH P(6-8)						R=0.628 P=0.022 N=13	R=0.571 P=0.041 N=13		R=0.604 P=0.029 N=13				
LH S(6-8)						R=0.825 P=0.000 N=14	R=0.870 P=0.009 N=14		R=0.641 P=0.014 N=14			R=0.553 P=0.040 N=14	
LH P(12-15)							R=0.535 P=0.049 N=14						
LH S(12-15)									R=0.661 P=0.014 N=13				
LH P(21-23)						R=0.812 P=0.000 N=14	R=0.555 P=0.039 N=14		R=0.702 P=0.005 N=14			R=0.534 P=0.049 N=14	
LH S(21-23)													
LH P(26-28)									R=0.670 P=0.009 N=14				
LH S(26-28)						R=0.573 P=0.041 N=13			R=0.732 P=0.004 N=13			R=0.648 P=0.017 N=13	
FSH													

P(1-3)													
FSH S(1-3)						R=0.706 P=0.005 N=14	R=0.820 P=0.000 N=14						
FSH P(6-8)						R=0.771 P=0.002 N=13	R=0.851 P=0.000 N=13					R=0.577 P=0.039 N=13	
FSH S(6-8)						R=0.744 P=0.002 N=14	R=0.866 P=0.000 N=14					R=0.540 P=0.046 N=14	
FSH P(12-15)						R=0.849 P=0.000 N=14	R=0.880 P=0.000 N=14					R=0.623 P=0.017 N=14	
FSH S(12-15)						R=0.796 P=0.001 N=13	R=0.826 P=0.001 N=13		R=0.614 P=0.026 N=13			R=0.767 P=0.002 N=13	
FSH P(21-23)						R=0.871 P=0.000 N=14	R=0.829 P=0.000 N=14		R=0.562 P=0.036 N=14			R=0.645 P=0.013 N=14	
FSH S(21-23)							R=0.635 P=0.015 N=14						
FSH P(26-28)				R=0.690 P=0.004 N=15						R=0.671 P=0.009 N=14	R=0.688 P=0.005 N=14	R=0.677 P=0.008 N=14	R=0.576 P=0.031 N=14
FSH S(26-28)						R=0.765 P=0.002 N=13	R=0.709 P=0.007 N=13		R=0.713 P=0.006 N=13			R=0.696 P=0.008 N=13	

Data compared using pearson correlation and presented (r ,P)

Table 5.A.11: Correlations of Estradiol and Progesterone with IGF-1, BP-3 and BP-1 in pre-menopausal women (n=16).

		Estradiol Placebo (6-8)	Estradiol Placebo (21-23)	Estradiol Supplement (12-14)	Estradiol Supplement (21-23)	Proges Supplement (1-3)	Proges Supplement (6-8)	Proges Supplement (12-14)
IGF-1(1-3)	placeb		-0.54, 0.031					
	supp				-0.64, 0.008			
IGF-1(6-8)	placeb							
	supp		-0.61, 0.012		-0.72, 0.002			
IGF-1(12-14)	placeb							
	supp				-0.76, 0.001			
IGF-1 (21-23)	placeb				-0.64, 0.008			
	supp				-0.65, 0.006			-0.55, 0.032
IGF-1 (26-28)	placeb							
	supp				-0.53, 0.04	-0.54, 0.038		-0.68, 0.008
BP-3 (1-3)	Placeb							
	supp				-0.51, 0.045			
BP-3 (6-8)	placeb			-0.60, 0.030				
	supp				-0.56, 0.023			
BP-3 (12-14)	Place							
	supp				-0.53, 0.041		-0.58, 0.025	
BP-3 (21-23)	Placeb							
	supp							
BP-3 (26-28)	Placeb							
	supp							
BP-1 (1-3)	placebo	0.61, 0.020				0.508, 0.045		0.69, 0.004
	supp	0.74, 0.003				0.79, 0.000		0.78, 0.001
BP-1 (6-8)	placebo	0.66, 0.010				0.63, 0.016		0.75, 0.003
	supp	0.68, 0.007			0.56, 0.023	0.65, 0.006		0.64, 0.01
BP-1 (12-14)	placebo	0.56, 0.04			0.53, 0.034	0.67, 0.006		0.71, 0.003
	supp				0.505, 0.055	0.59, 0.022		0.53, 0.043
BP-1 (21-23)	placebo	0.73, 0.003				0.55, 0.026		0.63, 0.009
	supp	0.69, 0.006			0.511, 0.043	0.69, 0.003		0.66, 0.008
BP-1 (26-28)	Placebo	0.69,0.006				0.61, 0.012		0.62, 0.013
	supp				0.63, 0.012	0.49, 0.063		0.63, 0.016

Data compared using pearson correlation and presented (r ,P).

Table 5.A.12: Correlations of SHBG with IGF-1 and BP-1 in pre-menopausal women (n=16).

		SHBG (Placebo phase)					SHBG (Supplement phase)				
		1-3	6-8	12-14	21-23	26-28	1-3	6-8	12-14	21-23	26-28
IGF-1(1-3)	placebo		-0.54, 0.036				-0.48, 0.061		-0.54, 0.036		-0.54, 0.039
	supp			-0.53, 0.044							
IGF-1(6-8)	placebo										
	supp		-0.55, 0.034	-0.56, 0.029					-0.54, 0.034		
IGF-1 (12-14)	placebo										
	supp			-0.57, 0.032							
	supp			-0.62, 0.014							
BP-1 (1-3)	placebo	0.55, 0.027	0.74, 0.002	0.73, 0.002	0.6, 0.014	0.61, 0.011	0.61, 0.012		0.58, 0.022	0.58, 0.017	0.60, 0.019
	supp	0.59, 0.016	0.75, 0.001	0.77, 0.001	0.63, 0.009	0.66, 0.005	0.60 0.015		0.59, 0.019	0.56, 0.023	0.62, 0.013
BP-1 (6-8)	placebo	0.62, 0.019	0.76, 0.001	0.74, 0.002	0.63, 0.016	0.65, 0.011	0.60, 0.023		0.63, 0.022	0.57, 0.034	0.61, 0.028
	supp	0.63, 0.009	0.78, 0.001	0.75, 0.001	0.67, 0.004	0.66 0.005	0.66, 0.005	0.51, 0.042	0.61, 0.017	0.61, 0.011	0.66, 0.008
BP-1 (12-14)	placebo	0.55, 0.027	0.62, 0.014	0.62, 0.014	0.56, 0.023	0.58, 0.019	0.49, 0.053				
	supp	0.50, 0.055	0.54, 0.050	0.57, 0.032	0.52, 0.048	0.57, 0.028	0.51, 0.051				
BP-1 (21-23)	placebo		0.68, 0.005	0.66, 0.007		0.53, 0036	0.54, 0.031		0.53, 0.043	0.50, 0.051	0.57, 0.026
	supp		0.59, 0.021	0.65, 0.09		0.50, 0.049					0.48, 0.07
BP-1 (26-28)	placebo		0.62, 0.013	0.56 0.037		0.50, 0.049					0.50, 0.06
	supp	0.55, 0.033	0.57, 0.032								

Data compared using pearson correlation and presented (r ,P)

Table 5.A.13: Correlations of hormones with IGF-1, BP-1 and BP-3 in post - menopausal women (n=7).

	LH (P1)	LH (S28)	Prog (P28)	Prog (S28)	SHBG (P1)	SHBG (P28)
BP1 (P1)	0.78, 0.037					
BP1 (S1)			0.75, 0.050			
BP3 (S1)		-0.78, 0.039				
IGF-1 (P1)					0.81, 0.028	0.77, 0.043
IGF-1 (P28)			-0.85, 0.016	-0.93, 0.0023		
IGF-1 (S28)					0.79, 0.033	

Data compared using pearson correlation and presented (r ,P)

Table 5.A.14: Correlations of serum concentrations of IGF-1 & BP-1 and Serum Lipids (no correlations seen with BP-3) in pre-menopausal women.

	HDL S(1-3)	HDL P(26-28)	HDL S(26-28)	Trig P(1-3)	Trig S(1-3)	Trig P(26-28)	Trig S(26-28)	Chol P(1-3)
BP1 S(1-3)	R=0.622 P=0.010 N=16	R=0.584 P=0.018 N=16	R=0.549 P=0.028 N=16					
BP1 S(6-8)	R=0.551 P=0.027 N=16				R=-0.523 P=0.038 N=16			
BP1 P(12-15)	R=0.555 P=0.026 N=16							
BP1 S(12-15)	R=0.756 P=0.001 N=15	R=0.533 P=0.041 N=15	R=0.629 P=0.012 N=15					
BP1 P(21-23)					R=-0.538 P=0.032 N=16			
BP1 S(21-23)	R=0.600 P=0.014 N=16							
BP1 S(26-28)	R=0.635 P=0.011 N=15		R=0.522 P=0.046 N=15					
IGF-1 P(1-3)				R=0.748 P=0.001 N=16	R=0.676 P=0.004 N=16		R=0.699 P=0.003 N=16	R=0.504 P=0.046 N=16
IGF-1 S(1-3)				R=0.510 P=0.027 N=16	R=0.822 P=0.000 N=16	R=0.565 P=0.022 N=16	R=0.538 P=0.032 N=16	
IGF-1 P(6-8)					R=0.608 P=0.023 N=14			
IGF-1 S(6-8)					R=0.576 P=0.019 N=16			
IGF-1 S(12-15)					R=0.671 P=0.006 N=15			
IGF-1 P(21-23)	R=-0.534 P=0.033 N=16	R=-0.509 P=0.044 N=16		R=0.713 P=0.002 N=16	R=0.594 P=0.015 N=16	R=0.502 P=0.048 N=16	R=0.678 P=0.004 N=16	
IGF-1 S(21-23)					R=0.604 P=0.013 N=16			
IGF-1 P(26-28)				R=0.550 P=0.027 N=16			R=0.504 P=0.047 N=16	
IGF-1 S(26-28)		R=-0.530 P=0.042 N=15	R=-0.528 P=0.043 N=15					

Data compared using pearson correlation and presented (*r* ,*P*)

Table 5.A.15: Correlations of serum concentrations of IGF1 with Serum antioxidants in pre menopausal women.

	IGF-1 P(1-3)	IGF-1 S(1-3)	IGF-1 P(6-8)	IGF-1 S(6-8)	IGF-1 P(12-15)	IGF-1 S(12-15)	IGF-1 P(21-23)	IGF-1 P(26-28)
Retinol P(1-3)	R=0.503 P=0.047 N=16	R=0.586 P=0.017 N=16						
Retinol S(1-3)	R=-0.655 P=0.006 N=16			R=-0.509 P=0.044 N=16			R=-0.591 P=0.016 N=16	R=-0.509 P=0.04 N=16
Vit C S(1-3)			R=-0.567 P=0.035 N=14					
Vit C S(26-28)			R=-0.540 P=0.046 N=14					
G- Tocoph P(1-3)	R=0.600 P=0.014 N=16							
G- Tocoph P(6-8)	R=-0.501 P=0.046 N=14							
A-Tocoph P(26-28)							R=0.623 P=0.010 N=16	
Lutein P(1-3)	R=0.503 P=0.047 N=16					R=0.533 P=0.041 N=15		
Zeaxanthyn P(1-3)						R=0.535 P=0.040 N=15		
Bcryp S(1-3)	R=-0.519 P=0.040 N=16				R=-0.557 P=0.025 N=16			
Bcryp P(26-28)					R=-0.495 P=0.050 N=16			
Lycopene S(1-3)	R=-0.557 P=0.025 N=16		R=-0.636 P=0.015 N=14	R=-0.610 P=0.012 N=16				
Lycopene P(26-28)			R=-0.585 P=0.028 N=14					
MDA P(1-3)	R=0.615 P=0.011 N=16				R=0.500 P=0.048 N=16			
MDA S(1-3)	R=0.533 P=0.033 N=16							

Data compared using pearson correlation and presented (r, P)

Table 5.A.16: Correlations of serum concentrations of BP1 and Serum antioxidants in premenopausal women.

	BP1 S(1-3)	BP1 P(6-8)	BP1 S(6-8)	BP1 S(21-23)	BP1 P(26-28)
B-Carotene S(1-3)	R=0.580 P=0.018 N=16	R=0.635 P=0.015 N=14	R=0.655 P=0.006 N=16	R=0.570 P=0.021 N=16	
B-Carotene S(26-28)	R=0.575 P=0.020 N=16	R=0.540 P=0.046 N=14			
A-Carotene S(1-3)	R=0.606 P=0.013 N=16	R=0.587 P=0.027 N=14	R=0.656 P=0.006 N=16	R=0.631 P=0.009 N=16	
A-Carotene P(26-28)			R=0.519 P=0.039 N=16		
A-Carotene S(26-28)	R=0.661 P=0.005 N=16	R=0.569 P=0.034 N=14			R=0.585 P=0.017 N=16
Lycopene S(1-3)		R=0.652 P=0.012 N=14	R=0.561 P=0.024 N=16		
A-Tocoph P(26-28)					R=0.532 P=0.034 N=16

Data compared using pearson correlation and presented (r ,P)

Table 5.A.17: Correlations of serum concentrations of BP-3 and Serum antioxidants in pre-menopausal women.

	BP3 P(1-3)	BP3 S(1-3)	BP3 P(6-8)	BP3 S(6-8)	BP3 P(12-14)	BP3 S(12-14)	BP3 P(21-23)	BP3 S(21-23)	BP3 P(26-28)	BP3 S(26-28)
Retinol S(1-3)		R=0.540 P=0.031 N=16						R=0.576 P=0.020 N=16	R=0.718 P=0.002 N=16	R=0.738 P=0.002 N=15
Retinol P(26-28)		R=0.635 P=0.008 N=16	R=0.626 P=0.017 N=14		R=0.499 P=0.049 N=16	R=0.586 P=0.022 N=15		R=0.589 P=0.016 N=16	R=0.638 P=0.008 N=16	R=0.697 P=0.004 N=15
Retinol S(26-28)										R=0.565 P=0.028 N=15
G-tocoph S(1-3)			R=0.589 P=0.027 N=14							
G-tocoph S(26-28)	R=0.552 P=0.027 N=16	R=0.520 P=0.039 N=16	R=0.633 P=0.015 N=14	R=0.50 P=0.049 N=16	R=0.584 P=0.017 N=16	R=0.573 P=0.026 N=15	R=0.519 P=0.039 N=16		R=0.574 P=0.020 N=16	R=0.536 P=0.040 N=15
a-tocoph S(1-3)									R=0.582 P=0.018 N=16	
Zeaxan P(26-28)							R=0.575 P=0.20 N=16			
B-cryp P(1-3)							R=0.598 P=0.014 N=16			
B-cryp P(26-28)			R=0.545 P=0.044 N=14		R=0.539 P=0.031 N=16	R=0.666 P=0.007 N=15	R=0.595 P=0.015 N=16	R=0.548 P=0.028 N=16	R=0.649 P=0.006 N=16	R=0.579 P=0.024 N=15
B-cryp S(1-3)			R=0.581 P=0.029 N=14			R=0.611 P=0.015 N=15			R=0.603 P=0.013 N=16	R=0.609 P=0.016 N=15
A-Caro P(26-28)									R=0.570 P=0.021 N=16	R=0.546 P=0.035 N=15
B-Caro P(26-28)									R=0.523 P=0.038	

									N=16	
Lyco P(26-28)							R=0.613 P=0.011 N=16			
MDA S(1-3)		R=-0.588 P=0.017 N=16		R=-0.519 P=0.039 N=16				R=-0.503 P=0.047 N=16		
MDA S(26-28)		R=-0.590 P=0.016 N=16	R=-0.702 P=0.005 N=14	R=-0.550 P=0.027 N=16	R=-0.641 P=0.007 N=16		R=-0.676 P=0.004 N=16		R=-0.527 P=0.036 N=16	R=-0.514 P=0.050 N=15

Data compared using pearson correlation and presented (r ,P)

Table 5.A.18: Correlations of serum concentrations of IGF-1, & BP-1 with anthropometric data in premenopausal women.
N.B post-menopausal correlations in last two rows

	Waist	Weight	Height	BMI	Hip
IGF P(1-3)	R=0.669 P=0.009 N=14	R=0.561 P=0.024 N=16		R=0.627 P=0.009 N=16	R=0.712 P=0.004 N=14
IGF-1 S(1-3)	R=0.538 P=0.047 N=14	R=0.626 P=0.009 N=16		R=0.527 P=0.036 N=16	R=0.615 P=0.019 N=14
IGF-1 P(6-8)	R=0.587 P=0.027 N=14				R=0.575 P=0.040 N=13
IGF-1 S(6-8)	R=0.501 P=0.048 N=16				
IGF-1 P(21-23)	R=0.560 P=0.024 N=16				R=0.551 P=0.041 N=14
IGF-1 S(21-23)			R=0.513 P=0.042 N=16		
IGF-1 S(26-28)		R=0.528 P=0.043 N=15			
BP1 P(1-3)		R=-0.54 P=0.031 N=16			
BP1 S(1-3)		R=-0.499 P=0.049 N=16			
BP1 P(6-8)	R=-0.557 P=0.048 N=13	R=-0.59 P=0.026 N=14		R=-0.627 P=0.016 N=14	R=-0.615 P=0.025 N=13
BP1 S(6-8)		R=-0.625 P=0.010 N=16		R=-0.586 P=0.017 N=16	R=-0.551 P=0.041 N=14
BP1 P(12-15)		R=-0.534 P=0.033 N=16			
BP1 P(21-23)		R=-0.604 P=0.013 N=16		R=-0.537 P=0.032 N=16	R=-0.600 P=0.023 N=14
BP1 S(21-23)		R=-0.642 P=0.007 N=16		R=-0.512 P=0.043 N=16	R=-0.512 P=0.043 N=16
BP1 S(26-28)		R=-0.639 P=0.010 N=15			
Postmen IGF-1 P(1-3)	R=-0.766 P=0.046 N=7				
Postmen BP-1 P(26-28)			R=0.803 P=0.030 N=7		

Data compared using pearson correlation and presented (r ,P)

Table 5.A.19:Correlations of BP-1 with serum lipids in post-menopausal women. N.B no correlations seen with BP-3 or IGF-1.

	BP1 P(1-3)	BP1 P(26-28)	BP1 S(26-28)
LDL P(1-3)		R=0.830 P=0.021 N=7	R=0.802 P=0.030 N=7
LDL P(26-28)		R=0.964 P=0.000 N=7	
LDL S(1-3)	R=0.792 P=0.034 N=7		R=0.782 P=0.038 N=7
LDL S(26-28)		R=0.899 P=0.006 N=7	
MDA P(26-28)			R=0.769 P=0.043 N=7
Chol P(1-3)			R=0.783 P=0.037 N=7
Chol P(26-28)		R=0.925 P=0.003 N=7	
Chol S(1-3)	R=0.782 P=0.038 N=7		

Data compared using pearson correlation and presented (r ,P).

Table 5.A.20: Correlations of IGF-1, BP-3 and BP-1 with serum antioxidants in postmenopausal women.

	Vit C P(1-3)	Vit C S(1-3)	Gtoc P(1-3)	Lutein P(26-28)	Zeax P(1-3)	Zeax P(26-28)
IGF P(1-3)	R=-0.862 P=0.013 N=7					
IGF P(26-28)	R=-0.776 P=0.040 N=7					
IGF S(26-28)	R=-0.760 P=0.048 N=7					
BP3 P(1-3)		R=-0.777 P=0.040 N=7				
BP3 P(26-28)			R=0.822 P=0.023 N=7			R=0.787 P=0.036 N=7
BP1 S(1-3)				R=-0.815 P=0.026 N=7	R=-0.775 P=0.041 N=7	R=-0.932 P=0.002 N=7
BP1 S(26-28)				R=-0.8000 P=0.031 N=7		

Data compared using pearson correlation.

Table 5.A.21: Correlations of Serum Lipid concentrations and FFQ/FD dietary intake in pre and postmenopausal women (n=23).

	Placebo					Supplement			
Serum Lipids	PUFA FFQ	Fat FFQ	MUFA FFQ	SFA FFQ	CHOL FFQ	PUFA FFQ	Fat FFQ	MUFA FFQ	%Fat
LDL Placebo/baseline	r= 0.461 P=0.041								
LDL Placebo/post	r=0.533 P=0.016								
LDL Supp/baseline	r=0.589 P=0.006	r=0.447 P=0.048							
Cholesterol Placebo/baseline	r=0.451 P=0.046								
Cholesterol Placebo/post	r=0.547 P=0.013	r=0.480 P=0.032	r=0.479 P=0.033						
Cholesterol Supp/baseline	r=0.585 P=0.007	r=0.506 P=0.023	r=0.455 P=0.044						
MDA Placebo/post		r=0.485 P=0.030	r=0.448 P=0.048	r=0.461 P=0.041	r=0.506 P=0.023				r=0.483 P=0.026
MDA Supp/baseline		r=0.460 P=0.041				r=0.522 P=0.015	r=0.515 P=0.017	r=0.437 P=0.048	

Data compared using pearson correlation and presented (r ,P)

Table 5.A.22: Correlations of Serum concentrations of antioxidants with FFQ/FD intake in pre and postmenopausal women (n=23). Serum vitamin C vs dietary vitamin C, Serum α and g tocopherol vs dietary Vitamin E and serum α and β carotene vs dietary Carotene.

	Vit C			Vit D			Vit E			Carotene				Biotin		Retinol
Serum	P-ffq	P-fd	S-fd	S-ffq	P-fd	S-fd	S-ffq	P-fd	S-fd	P-ffq	S-ffq	P-fd	S-fd	P-ffq	S-ffq	P-ffq
Vit C P-base										R=0.476 P=0.034		R=0.494 P=0.027				
Vit C S-base	R=0.450 P=0.046															
Vit C S-post					R=0.476 P=0.034					R=0.524 P=0.021						
Retinol P-base			R=-0.46 P=0.045		R=0.530 P=0.016											
G-toc P-base					R=0.657 P=0.002	R=0.502 P=0.029		R=0.481 P=0.032								
G-toc P-post				R=0.624 P=0.002					R=0.472 P=0.041							
G-toc S-base																
G-toc S-post																R=0.468 P=0.037
a-toc P-base					R=0.631 P=0.003	R=0.634 P=0.004										
a-toc P-post									R=0.476 P=0.039							
a-toc S-base																
a-toc S-post															R=0.452 P=0.040	
Lutein P-post										R=0.716 P=0.000		R=0.613 P=0.004				
Lutein S-base										R=0.702 P=0.001		R=0.513 P=0.021				
Lutein S-post		R=0.458 P=0.042								R=0.507 P=0.022		R=0.69 P=0.001	R=0.54 P=0.017	R=0.522 P=0.018		
Zeax					R=0.445							R=0.485				

P-base					P=0.049							P=0.03				
Zeax P-post										R=0.662 P=0.001						
Zeax S-base										R=0.654 P=0.002						
Zeax S-post										R=0.506 P=0.023						
Bcryp P-post														R=0.475 P=0.034	R=0.451 P=0.040	
Bcryp S-base												R=0.488 P=0.029				
Acaro P-base												R=0.635 P=0.003				
Acaro P-post												R=0.504 P=0.024				
Acaro S-base										R=0.487 P=0.029		R=0.682 P=0.001				
Acaro S-post												R=0.692 P=0.001	R=0.550 P=0.015			
Bcaro P-base												R=0.610 P=0.004				
Bcaro P-post												R=0.547 P=0.013				
Bcaro S-base												R=0.711 P=0.000	R=0.457 P=0.049			
Bcaro S-post												R=0.642 P=0.002	R=0.522 P=0.022	R=0.447 P=0.048		
Lyco P-post										R=0.542 P=0.014						
Lyco S-base										R=0.629 P=0.003						
Lyco S-post				R=0.627 P=0.002			R=0.50 P=0.022			R=0.468 P=0.038						

Data compared using pearson correlation and presented (r ,P)

Table 5.A.23: Correlations of IGF-1 concentrations with FFQ/FD intake in pre-menopausal women.

	IGF-1 P(1-3)	IGF-1 S(1-3)	IGF-1 P(6-8)	IGF-1 S(6-8)	IGF-1 S(12-14)	IGF-1 P(21-23)	IGF-1 S(21-23)	IGF-1 P(26-28)
Carotene S FFQ			R=0.657 P=0.015 n=13					
Vit B6 S FFQ		R=0.604 P=0.022 n=14					R=0.545 P=0.044 n=14	
Vit B12 S FFQ								R=0.537 P=0.048 n=14
Vit B12 S FD	R=0.694 P=0.012 n=12			R=0.606 P=0.037 N=12				
Biotin S FFQ		R=0.627 P=0.016 n=14		R=0.691 P=0.009 n=13				
Vit C S FFQ			R=0.610 P=0.027 n=13		R=0.594 P=0.025 n=14	R=0.557 P=0.038 n=14		
PUFA S FFQ			R=0.637 P=0.019 n=13					R=0.574 P=0.032 n=14
Fat P FD								R=-0.652 P=0.012 N=13
SFA P FD								R=-0.612 P=0.026 N=13
MUFA P FD								R=-0.646 P=0.017 N=13
Energy P FD								R=-0.581 P=0.037 N=13
Starch P FD								R=-0.567 P=0.043 N=13
Sodium P FD						R=-0.621 P=0.023 N=13		R=-0.729 P=0.005 N=13
Copper P FD							R=-0.566 P=0.044 N=13	R=-0.631 P=0.021 N=13
Chloride P FD								R=-0.645 P=0.017 N=13
Selenium P FD						R=-0.602 P=0.029 N=13		R=-0.628 P=0.022 N=13
Vit D P FD		R=0.625 P=0.022 N=13			R=0.605 P=0.037 N=12			
Vit D S FD		R=0.755 P=0.005 N=12				R=0.592 P=0.042 N=12		R=0.630 P=0.028 N=12
Vit E S FD			R=-0.671 P=0.024 N=11	R=-0.587 P=0.045 N=12				
Fibre (Englyst) S FD	R=-0.725 P=0.008 N=12							

Data compared using pearson correlation and presented (r ,P)

Table 5.A.24: Correlations of BP-1 concentrations with FFQ/FD intake in pre-menopausal women.

	BP1 P(1-3)	BP1 S(1-3)	BP1 P(6-8)	BP1 S(6-8)	BP1 P(12-14)	BP1 S(12-14)	BP1 P(21-23)	BP1 S(21-23)	BP1 P(26-28)	BP1 S(26-28)
Carotene P FFQ								R=0.557 P=0.048 N=13		
%Carbo P FFQ						R=0.668 P=0.018 N=12				
Protein S FFQ							R=-0.558 P=0.027 N=14			
Magnesium S FFQ							R=-0.575 P=0.031 N=14			
Phosphorous S FFQ							R=-0.556 P=0.039 N=14			
Tryptophan S FFQ							R=-0.578 P=0.030 N=14			
Water P FD										R=-0.585 P=0.045 N=12
Manganese P FD						R=-0.588 P=0.044 N=12				
Selenium S FD							R=-0.634 P=0.027 N=12			
Iodine S FD	R=-0.597 P=0.041 N=12					R=-0.607 P=0.022 N=12			R=-0.651 P=0.022 N=12	
Vit E S FD	R=0.626 P=0.029 N=12	R=0.635 P=0.027 N=12	R=0.742 P=0.009 N=11	R=0.7221 P=0.008 N=12	R=0.745 P=0.005 N=12		R=0.649 P=0.002 N=12	R=0.608 P=0.036 N=12		
Vit B12 S FD						R=-0.655 P=0.021 N=12	R=-0.593 P=0.041 N=12			R=-0.664 P=0.026 N=11
Vit B6 P FFQ						R=0.578 P=0.049 N=12				

Data compared using pearson correlation and presented (r, P)

Table 5.A.25: Correlations of BP-3 concentrations with FFQ/FD intake in pre-menopausal women.

	BP3 P(1-3)	BP3 S(1-3)	BP3 P(6-8)	BP3 S(6-8)	BP3 P(12-14)	BP3 S(12-14)	BP3 P(21-23)	BP3 S(21-23)	BP3 P(26-28)	BP3 S(26-28)
Carbo P FFQ		R=-0.632 P=0.021 N=13	R=-0.577 P=0.049 N=12	R=-0.647 P=0.017 N=13	R=-0.580 P=0.038 N=13				R=-0.631 P=0.021 N=13	
Starch S FD			R=-0.705 P=0.015 N=11							
Sugar P FFQ		R=-0.565 P=0.044 N=13					R=-0.640 P=0.018 N=13			
Fibre Southgate P FFQ	R=-0.643 P=0.018 N=13	R=-0.658 P=0.015 N=13		R=-0.674 P=0.012 N=13	R=-0.621 P=0.023 N=13	R=-0.611 P=0.035 N=12		R=-0.577 P=0.039 N=13		
Fibre Southgate S FFQ				R=-0.546 P=0.043 N=14	R=-0.566 P=0.035 N=14		R=-0.553 P=0.040 N=14			
Fibre Englyst P FFQ	R=-0.587 P=0.035 N=13	R=-0.597 P=0.031 N=13		R=-0.560 P=0.047 N=13						
Fibre Englyst S FFQ				R=-0.541 P=0.046 N=14						
Fibre Southgate S FD	R=-0.632 P=0.028 N=12									
Calcium S FD	R=-0.725 P=0.008 N=12				R=-0.694 P=0.012 N=12		R=-0.688 P=0.013 N=12			
Magnesium P FFQ	R=-0.560 P=0.046 N=13							R=-0.567 P=0.043 N=13		
Magnesium S FFQ							R=-0.548 P=0.043 N=14			
Phosphorous S FD	R=-0.720 P=0.008 N=12						R=-0.684 P=0.014 N=12		R=-0.621 R=0.031 N=12	

Zinc S FD	R=-0.578 P=0.049 N=12									
Manganese S FFQ							R=-0.556 P=0.039 N=14			
Copper S FFQ							R=-0.545 P=0.044 N=14			
Vit D P FFQ									R=0.558 P=0.047 N=13	
% Carbo P FFQ			R=-0.581 P=0.048 N=12				R=-0.634 P=0.020 N=13		R=-0.651 P=0.016 N=13	
% Fat P FFQ						R=0.584 P=0.046 N=12			R=0.616 P=0.025 N=13	
% Protein S FFQ					R=0.536 P=0.048 N=14					
% Protein S FD										R=-0.636 P=0.035 N=11
Chol S FFQ										R=0.608 P=0.027 N=13
Chol S FD	R=-0.657 P=0.020 N=12					R=-0.600 P=0.039 N=12	R=-0.644 R=0.024 N=12		R=-0.692 P=0.013 N=12	R=-0.629 P=0.038 N=11
Vit E S FFQ			R=-0.632 P=0.021 N=13		R=-0.560 P=0.037 N=14		R=-0.583 P=0.029 N=14			
Vit C P FD					R=0.608 P=0.028 N=13			R=0.555 P=0.049 N=13		
Protein S FD	R=-0.694 P=0.012 N=12							R=-0.595 P=0.041 N=12		R=-0.666 P=0.025 N=11
Carbo					R=-0.625					

S FD					P=0.030 N=12					
Nitrogen S FD	R=-0.693 P=0.013 N=12							R=-0.585 P=0.046 N=12		R=-0.656 P=0.028 N=11
Chloride S FD	R=-0.584 P=0.046 N=12		R=-0.621 P=0.041 N=11		R=-0.579 P=0.048 N=12					
Selenium S FD	R=-0.616 P=0.033 N=12	R=-0.650 P=0.022 N=12			R=-0.613 P=0.034 N=12		R=-0.602 P=0.038 N=12	R=-0.722 P=0.008 N=12		R=-0.708 P=0.015 N=11
Tryptophan S FD	R=-0.674 P=0.016 N=12						R=-0.637 P=0.026 N=12	R=-0.595 P=0.041 N=12	R=-0.585 P=0.046 N=12	R=-0.622 P=0.041 N=11
Energy Kcal S FD	R=-0.688 P=0.013 N=12									
Sodium S FD	R=-0.602 P=0.038 N=12									
Fat S FD	R=-0.741 P=0.006 N=12									
SFA S FD	R=-0.762 P=0.004 N=12									
MUFA S FD	R=-0.672 P=0.017 N=12									

Data compared using pearson correlation and presented (r ,P)

Table 5.A.26: Correlations of IGF-1 with FFQ/FD in post-menopausal women

	IGF-1 P(1-3)	IGF-1 S(1-3)	IGF-1 P(26-28)	IGF-1 S(26-28)
Water PFFQ	R=-0.759 P=0.048 N=7			R=-0.861 P=0.013 N=7
Water SFFQ				R=-0.828 P=0.021 N=7
Protein PFFQ			R=-0.916 P=0.004 N=7	
Protein SFFQ	R=-0.809 P=0.028 N=7		R=-0.953 P=0.001 N=7	R=-0.840 P=0.018 N=7
Fat PFFQ			R=-0.825 P=0.022 N=7	
Carbo PFFQ			R=-0.788 P=0.035 N=7	
Energy PFFQ			R=-0.961 P=0.001 N=7	
Energy SFFQ		R=-0.846 P=0.016 N=7		R=-0.863 P=0.012 N=7
Nitrogen PFFQ			R=-0.960 P=0.003 N=7	
Nitrogen SFFQ	R=-0.820 P=0.024 N=7		R=-0.961 P=0.001 N=7	R=-0.836 P=0.019 N=7
MUFA PFFQ			R=-0.932 P=0.002 N=7	
PUFA SFFQ			R=-0.810 P=0.027 N=7	
Starch PFFQ			R=-0.903 P=0.005 N=7	
Starch PFD				R=0.793 P=0.034 N=7
Fibre (south) PFD				R=0.768 P=0.044 N=7
Fibre (engly) PFD	R=0.813 P=0.026 N=7			
Sodium PFFQ			R=-0.865 P=0.012 N=7	
Potass PFFQ				R=-0.777 P=0.040 N=7
Potass SFFQ		R=-0.809 P=0.028 N=7		R=-0.784 P=0.037 N=7
Calcium				R=-0.818

SFFQ				P=0.025 N=7
Phosph PFFQ	R=-0.814 P=0.026 N=7		R=-0.979 P=0.000 N=7	R=-0.791 P=0.034 N=7
Phosph SFFQ	R=-0.785 P=0.037 N=7		R=-0.830 P=-0.021 N=7	R=-0.898 P=0.006 N=7
Iron PFFQ			R=-0.876 P=0.010 N=7	
Copper PFD				R=0.776 P=0.040 N=7
Zinc PFFQ	R=-0.792 P=0.034 N=7		R=-0.971 P=0.000 N=7	
Zinc SFFQ			R=-0.870 P=0.011 N=7	R=-0.871 P=0.011 N=7
Chloride PFFQ			R=-0.890 P=0.007 N=7	
Mangan PFFQ			R=-0.755 P=0.050 N=7	
Mangan PFD				R=0.795 P=0.033 N=7
Selen PFD				R=0.772 P=0.042 N=7
Iodine SFFQ				R=-0.827 P=0.022 N=7
Thiamin PFFQ			R=-0.875 P=0.010 N=7	
Riboflav PFFQ			R=-0.779 P=0.039 N=7	
Niacin PFFQ			R=-0.846 P=0.016 N=7	
Tryptop PFFQ			R=-0.901 P=0.006 N=7	
Tryptop SFFQ	R=-0.799 P=0.031 N=7		R=-0.949 P=0.001 N=7	R=-0.826 P=0.022 N=7
Vit B6 SFFQ	R=-0.876 P=0.010 N=7		R=-0.794 P=0.033 N=7	R=-0.885 P=0.008 N=7
Pantoth PFFQ			R=-0.874 P=0.010 N=7	
Pantoth SFFQ	R=-0.816 P=0.025 N=7		R=-0.945 P=0.001 N=7	R=-0.874 P=0.010 N=7

Data compared using pearson correlation and presented (r ,P)

Table 5.A.27: Correlations of BP-3 and BP-1 with FFQ/FD in post-menopausal women

	BP3 P(1-3)	BP3 S(1-3)	BP3 P(26-28)	BP3 S(26-28)	BP1 P(1-3)	BP1 S(1-3)	BP1 P(26-28)	BP1 S(26-28)
Water SFD					R=-0.843 P=0.017 N=7			
Fat PFFQ						R=0.878 P=0.009 N=7		
Fat SFFQ						R=0.800 P=0.031 N=7		
Carbo PFFQ						R=0.760 P=0.048 N=7		
Energy PFFQ						R=0.869 P=0.011 N=7		
Energy SFFQ	R=-0.791 P=0.034 N=7		R=-0.807 P=0.028 N=7	R=-0.755 P=0.05 N=7		R=0.780 P=0.039 N=7		
Nitrogen SFD					R=-0.758 P=0.049 N=7			R=-0.758 P=0.048 N=7
SFA PFFQ			R=-0.775 P=0.041 N=7	R=-0.779 P=0.039 N=7				
MUFA PFFQ						R=0.803 P=0.030 N=7		
MUFA SFFQ						R=0.755 P=0.050 N=7		
PUFA SFFQ						R=0.804 P=0.029 N=7		
Chol PFD					R=-0.795 P=0.033 N=7			
Starch PFFQ						R=0.850 P=0.015 N=7		
Starch SFD					R=-0.785 P=0.037 N=7			
Sugar SFFQ		R=-0.804 P=0.029 N=7		R=-0.755 P=0.050 N=7				
Sugar SFD			R=-0.762 P=0.047 N=7					
Fibre (south) PFD							R=-0.764 P=0.046 N=7	
Fibre (south) SFD								R=-0.853 P=0.015 N=7
Sodium PFFQ						R=0.852 P=0.015 N=7		
Sodium PFD					R=-0.861 P=0.013 N=7			
Sodium SFD					R=-0.802 P=0.030 N=7			R=-0.755 P=0.050 N=7
Potass SFFQ		R=-0.755 P=0.050 N=7						
Potass SFD								R=-0.831 P=0.020 N=7
Magnes								R=-0.792 P=0.034

SFD								N=7
Phos SFD								R=-0.771 P=0.042 N=7
Iron SFD								R=-0.850 P=0.015 N=7
Copper SFFQ						R=-0.833 P=0.020 N=7		R=-0.757 P=0.049 N=7
Copper SFD					R=-0.840 P=0.018 N=7			R=-0.811 P=0.027 N=7
Chloride PFFQ						R=0.855 P=0.014 N=7		
Chloride PFD					R=-0.855 P=0.014 N=7			
Chloride SFD					R=-0.772 P=0.042 N=7			
Selenium PFFQ						R=0.902 P=0.005 N=7		R=0.755 P=0.050 N=7
Iodine SFFQ						R=0.763 P=0.046 N=7		
Iodine SFD								R=-0.766 P=0.044 N=7
Carotene PFFQ					R=-0.774 P=0.041 N=7			R=-0.760 P=0.047 N=7
Carotene SFFQ					R=-0.826 P=0.022 N=7			
Carotene PFD								R=-0.777 P=0.040 N=7
Carotene SFD								R=-0.795 P=0.033 N=7
Vit E SFFQ							R=0.817 P=0.025 N=7	
Vit E SFD		R=0.767 P=0.044 N=7						
Riboflavin SFD								R=-0.791 P=0.034 N=7
Niacin SFD								R=-0.913 P=0.004 N=7
Folate PFD							R=-0.763 P=0.046 N=7	
Folate SFD								R=-0.874 P=0.010 N=7
Vit B6 PFFQ							R=0.836 P=0.019 N=7	
Biotin PFD								R=-0.938 P=0.002 N=7
Vit C PFD							R=-0.756 P=0.049 N=7	
%carbo SFD					R=0.803 P=0.03 N=7			
%Fat PFD					R=-0.804 P=0.029 N=7			

Data compared using pearson correlation and presented (*r* ,*P*

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